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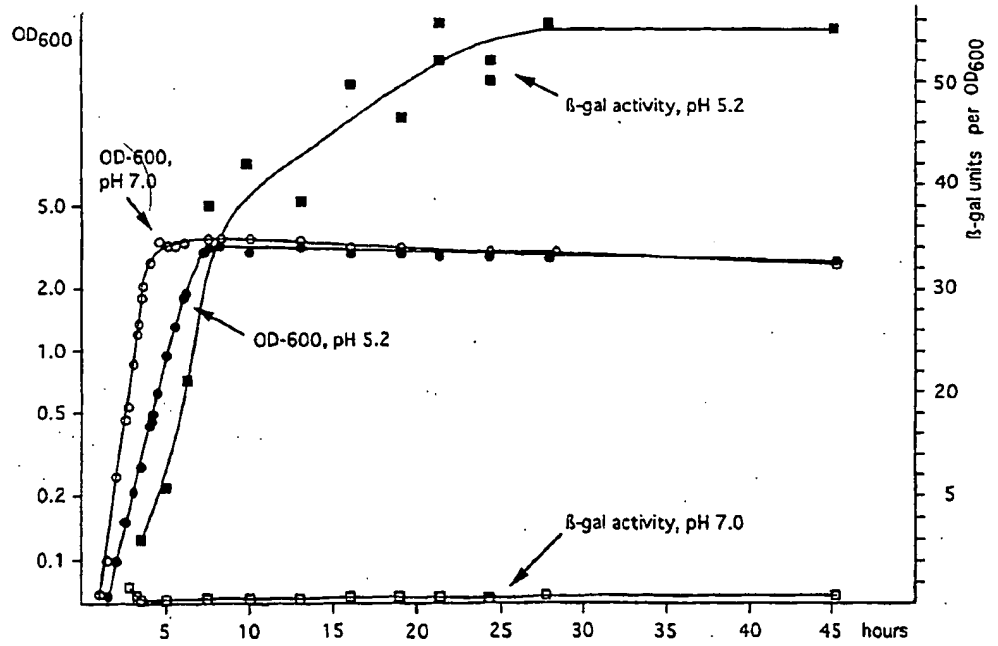

(57) Abstract

A method of isolating a lactic acid bacterial DNA fragment comprising a promoter, the method comprising introducing a DNA molecule comprising a transposable element comprising a promoterless structural gene as a promoter probe gene into a population of a lactic acid bacterium, methods of constructing a recombinant lactic acid bacterium comprising a regulatable promoter by using the above method, a recombinant lactic acid bacterium comprising a gene coding for a desired gene product and operably linked thereto a regulatable lactic acid bacterial promoter not natively associated with the gene, the use of such a recombinant lactic acid bacterium and recombinant plasmids comprising a regulatable lactic acid bacterial promoter.

RECOMBINANT LACTIC ACID BACTERIUM CONTAINING AN INSERTED PROMOTER AND METHOD OF CONSTRUCTING SAME

FIELD OF INVENTION

This invention pertains to the field of genetically improved food grade lactic acid bacteria. In particular there are provided methods for isolating useful lactic acid bacterial promoters and construction of recombinant lactic acid bacteria in which such promoters are utilized to obtain improved lactic acid bacteria which are useful in the manufacturing of foods, animal feed and probiotically active compositions.

TECHNICAL BACKGROUND AND PRIOR ART

For centuries, lactic acid bacterial cultures have been used in food production due to their ability to convert sugars by fermentation into preserving organic acids, predominantly lactic acid, and various metabolites associated with the development in fermented food products of desirable taste and flavour. Several lactic acid bacteria produce hydrolytic enzymes including peptidases, proteases and lipolytic enzymes, the production of which may e.g. contribute to a desired flavour development in cheeses.

However, for industrial production of a wide range of fermented food products such as all the well-known traditional dairy products including yoghurt, acidophilus milk, butter and cheeses; fermented vegetables; fermented meat products and animal feed, a large range of lactic acid bacterial starter cultures, each being adapted to particular types of food products, are required. Such cultures are presently being selected from naturally occurring strains of lactic acid bacteria on the basis of characteristics such as their ability to ferment sugars present in the food product to be

fermented, specific growth temperature requirements, production of desired flavouring compounds, the specific combination of which characteristics renders a specifically selected wild-type culture useful for the production of a particular food product but normally less useful for the production of others.

Obviously, this presently used procedure for developing useful lactic acid bacterial cultures by selection of naturally occurring strains is cumbersome and costly. Furthermore, it has proven difficult to provide starter culture strains which combine all of the required characteristics at an optimal level. Presently, this problem is usually solved by the use of starter cultures comprising a multiplicity of selected lactic acid bacterial strains each having one or several of the characteristics desirable for a particular food product. The necessity to use such mixed cultures will of course add to the costs in the manufacture of lactic acid bacterial starter cultures.

Based on their traditional and long term application in food manufacturing and the fact that they are considered as non-pathogenic, the lactic acid bacteria are generally recognized as safe (GRAS) food ingredients, even if they are present in a fermented food product as live bacteria at a very high number, such as 10^8 to 10^9 per g.

Currently, it is widely recognized that a substantial industrial need exists to find economically and technically more feasible ways of developing starter cultures. It is obvious that gene technology may provide the means to meet this need. In this context, it is crucial that lactic acid bacteria for food manufacturing which are developed by introduction of desired genes by use of gene technology can still by recognized as safe for consumption. It is therefore considered by the industry that it is essential that recombinant lactic acid bacteria contain only DNA of lactic acid bacterial origin including DNA from wild-type extrachromosomal plasmids

frequently found in starter culture strains or non-lactic acid bacterial DNA which does not confer to the recombinant strains any hazardous phenotypic traits.

There have been several attempts of providing genetically improved lactic acid bacteria. Most of these attempts have been directed to the construction of recombinant expression vectors coding for desired gene products and capable of replicating in lactic acid bacteria. However, very few of these attempts have resulted in vectors comprising only lactic acid bacterial DNA.

Another approach to the improvement of lactic acid bacteria would be to have useful genes inserted into the chromosome of the bacteria or to enhance the expression of chromosomal genes coding for desired gene products. Such an approach might, if successful, circumvent the problem which is frequently encountered when new genes are introduced on a plasmid, viz. the loss of such plasmids due to inherent instability or as a result of the presence of other plasmids belonging to a different incompatibility group. In contrast thereto, an introduced gene which becomes integrated in the chromosome is generally stably inherited by daughter cells.

However, this latter approach is still not well-studied in lactic acid bacteria due to the lack of detailed knowledge of the chromosomes of lactic acid bacteria and due to lack of suitable methods of obtaining chromosomal integration of heterologous DNA, although recent publications have reported on such chromosomal integration in *Lactococcus lactis* ssp. *lactis* by means of so-called integration vectors (reference 46)

It is known that the expression of a homologous or heterologous gene may be enhanced, e.g. by replacing a promoter sequence naturally associated with that gene with a stronger promoter sequence which results in an enhanced expression of the gene at the transcriptional level. Thus, DD 228 564 dis-

closes a method of preparing an expression vector capable of replication in *E. coli* and/or *B. subtilis*, comprising inserting into a unique restriction site a promoterless basic *E. coli* and/or *B. subtilis* plasmid comprising a structural gene, a promoter-carrying DNA fragment isolated from a *Streptococcus* species by restriction with a restriction enzyme corresponding to the unique restriction site of the basic plasmid, and isolating the thus recombinant vector from *E. coli* and/or *B. subtilis* transformed with the vector and expressing the structural gene.

Youngman et al. (1987) disclosed a method for the isolation of promoters in *Bacillus* spp. using the transposon Tn*917*. However, this method is based on the ability of *Bacillus* spp. to grow at temperatures above 37°C and it has furthermore been found that this transposition procedure in *Bacillus* spp. results in the transposon being integrated into a dominating hot spot whereby a single dominant integrant will occur.

It has recently been suggested that sequences comprising a lactic acid bacterial promoter and/or promoter-signal peptide sequences may be used to replace weaker native promoters and/or promoter-signal peptide sequences in plasmids to obtain a more efficient expression and secretion of an *E.coli* gene product, viz. β-lactamase in the lactic acid bacterium *Lactococcus lactis*. (reference 28). These authors identified the *Lactococcus* promoter sequences by means of a promoter probe vector capable of replication in *E. coli* and/or *B. subtilis* and comprising a promoterless *cat* gene and suitable restriction sites into which fragments of the *Lactococcus* chromosome could be inserted followed by screening for recombinant plasmids isolated from *E. coli* or *B. subtilis* and expressing the *cat* gene.

However, such a method involving the screening in a non-lactic acid bacterium for insertion of lactic acid bacterial promoters in a vector which is not of lactic acid bacterial origin and which is replicated in a non-lactic acid bacterium

does not allow for a direct *in situ* identification of a useful lactic acid bacterial promoter while functioning in the lactic acid bacterium of origin. Such a direct method is provided by the present invention.

SUMMARY OF THE INVENTION

In one aspect the present invention relates to a method of isolating a lactic acid bacterial DNA fragment comprising a promoter, the method comprising the steps of:

(i) selecting a DNA molecule replicating in a lactic acid bacterium, said molecule comprising (a) a transposable element comprising a promoterless structural gene as a promoter probe gene, (b) a detectable selective marker gene, and (c) an origin of replication which is functional in a lactic acid bacterium,

(ii) introducing the DNA molecule into a population of a lactic acid bacterium, followed by subjecting the population to conditions allowing transposition of the transposable element to occur,

(iii) selecting a cell of the lactic acid bacterial population in which the promoterless gene is expressed,

(iv) cloning said cell and isolating from the clone a DNA fragment comprising a lactic acid bacterial promoter being operably linked to the originally promoterless gene and possibly sequences regulating the function of the promoter.

In further aspects the invention provides methods of constructing a recombinant lactic acid bacterium comprising the steps of:

(i) isolating in accordance with the above method a DNA fragment comprising a regulatable lactic acid bacterial promoter,

(ii) inserting the isolated fragment comprising the promoter into a lactic acid bacterium upstream of a gene coding for a desired gene product, the inserted promoter thereby becoming operably linked to said gene or

a method of constructing a recombinant lactic acid bacterium comprising the steps of:

(i) isolating in accordance with the method of claim 1 a DNA fragment comprising a regulatable lactic acid bacterial promoter,

(ii) inserting into a lactic acid bacterium a gene coding for a desired gene product,

(iii) inserting the isolated fragment comprising the promoter into the lactic acid bacterium resulting from step (ii) upstream of the gene coding for a desired gene product, the inserted promoter thereby becoming operably linked to said gene.

In a still further aspect, the invention relates to a method of constructing a recombinant lactic acid bacterium comprising the steps of:

(i) selecting a DNA molecule replicating in a lactic acid bacterium, said molecule comprising (a) a transposable element comprising a promoterless structural gene as a promoter probe gene, (b) a detectable selective marker gene, and (c) an origin of replication which is functional in a lactic acid bacterium,

(ii) introducing under conditions allowing transposition of the transposable element to occur, the DNA molecule of step (i) into a population of a lactic acid bacterium,

(iii) selecting a cell of the lactic acid bacterial population in which the promoterless structural gene is regulatably expressed as a result of being operably linked to a native regulatable promoter of the lactic acid bacterial cell,

(iv) identifying the site in a replicon of the lactic acid bacterial cell of step (iii) into which the transposable element is integratable, and

(v) inserting into a non-integrant cell of the lactic acid bacterial population at a site as identified in step (iv) or at a functionally equivalent site, a gene coding for a desired gene product whereby the gene becomes operably linked to said native lactic acid bacterial promoter,

the expression of the inserted gene hereby being altered as compared to the expression of the gene when operably linked to its native promoter.

In other further aspects the present invention relates to a recombinant lactic acid bacterium comprising a gene coding for a desired gene product and operably linked thereto a regulatable lactic acid bacterial promoter not natively associated with the gene, the presence of said promoter resulting in the expression of the gene being altered as compared to the expression of the gene when operably linked to its native promoter and to an isolated DNA fragment comprising a lactic acid bacterial promoter which is functional in a lactic acid bacterium and operably linked thereto, a gene coding for a desired gene product, said promoter being one which is not naturally associated with the gene.

The invention also relates to the use of a recombinant lactic acid bacterium as defined herein in the manufacturing of food products, in the preservation of animal feed and in the manufacturing of a probiotically active composition.

In yet an other aspect the present invention provides a recombinant plasmid comprising a DNA sequence comprising a regulatable lactic acid bacterial promoter, a gene coding for a desired gene product, a lactic acid bacterial replicon which is functional in a lactic acid bacterium, an insertion site allowing the DNA sequence to be inserted so that the gene coding for the desired gene product is operably linked to the promoter, whereby the gene can be transcribed when the plasmid is present in a lactic acid bacterium.

In a still further aspect, the present invention relates to a recombinant plasmid comprising a vector comprising a promoterless gene coding for a desired gene product, a theta-replicating lactic acid bacterial replicon which is functional in a lactic acid bacterium and an insertion site allowing a DNA sequence to be inserted, and inserted into said insertion site a DNA sequence comprising a regulatable lactic acid bacterial promoter, the insertion resulting in that the gene coding for the desired gene product is operably linked to the promoter, whereby the gene is transcribed. Thus, such a plasmid may comprise as the vector, the plasmid pAK80.

DETAILED DISCLOSURE OF THE INVENTION

A primary object of the present invention is to provide the means of constructing improved lactic acid bacteria which are food grade in the sense that they contain only DNA derived from a lactic acid bacterial species or DNA from a non-lactic acid bacterial species the presence of which may be generally recognized as safe. As used herein the term "lactic acid bacterium" designates gram-positive, microaerophilic or anaerobic bacteria which ferment sugars with the production

of acids including lactic acid as the predominantly produced acid, acetic acid and propionic acid. The industrially most useful lactic acid bacteria are found among *Lactococcus* spp., *Streptococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* spp., *Brevibacterium* spp., *Propionibacterium* spp. and *Bifidobacterium* spp.

As it is mentioned above, the invention provides in one aspect a method of isolating a lactic acid bacterial DNA fragment comprising a promoter. In a first step of this method there is provided a DNA molecule capable of replicating in a lactic acid bacterium, said molecule comprising a transposable element, a promoterless structural gene as a promoter probe gene, a detectable selective marker gene, and an origin of replication which is functional in a lactic acid bacterium. Provided such a fragment can be introduced into a lactic acid bacterium and subsequently become integrated in a host cell replicon (including the chromosome and/or plasmids carried by the host) as a result of transposition events, host cell promoters may be identified by the detection of expression in the host cell of the promoterless structural gene of the integrated DNA fragment, since the structural gene lacking a promoter region cannot be expressed unless the insertion of the transposable element occurs at a site of a replicon where a promoter region present on the disrupted replicon molecule becomes operably linked to the gene.

In the present context, the term "transposable element" is used to designate double stranded DNA molecules which possess the capacity to insert themselves into other DNA molecules. The process by which a transposable element inserts itself is termed "transposition" and this process requires a protein known as a "transposase" (cf. reference 3 for detailed explanations). The transposition process results in the insertion of the transposable element into a particular site in a second DNA molecule. This insertion has several significant consequences. First, the original DNA sequence of the second (recipient) DNA molecule is physically and functionally

disrupted. Second, since transposition results in the incorporation of new DNA into a second DNA molecule, it provides the means of introducing homologous or heterologous DNA into a particular DNA sequence. Third, it is possible to engineer a transposable element so that its insertion into a DNA sequence can provide information regarding the expression and organization of the DNA sequence which flank the site of insertion. For example, it is possible to insert a gene which encodes a non-expressed or non-excreted gene product near the end of a transposable element and accordingly, such a transposable element provides a probe for promoters and secretion signal peptide.

Transposable elements which may be used in accordance with the invention are diverse in both size and functional organization. Thus, simple transposable elements, termed "insertion sequences", encode no functions unrelated to their own movement and are generally shorter than 2 kb. Like all transposable elements, insertion sequences possess specialized termini which contain complementary sequences which are inverted repeats of one another. The presence of such inverted repeat sequences appears to be essential for transposition. Transposase enzymes are thought to mediate transposition by binding to DNA sequences at both ends of the transposable element.

Useful transposable elements include transposons. The term "transposons" denotes transposable elements which are larger than insertion sequences and which in addition to the transposase system encode several gene products such as proteins which confer cellular resistance to antibiotics or other selectable determinants.

Although most work concerning the exploitation of transposable elements as gene technology tools has been done in gram-negative bacterial species, several transposons which are functional in gram-positive species have been isolated and studied, mainly in *Bacillus* spp, *Listeria* spp and *Coryne-*

bacterium spp, but also to less extent in lactic acid bacteria. Examples of transposons which may be used in lactic acid bacteria include Tn*916* isolated from *Streptococcus* and functional i.a. in *Listeria* spp, *Mycoplasma* spp, *Staphylococcus* spp; Tn*919* isolated from *Streptococcus sanguis* which has been shown to transpose in the lactic acid bacterial species *Lactobacillus plantarum*, *Leuconostoc cremoris* and *Lactococcus lactis*; and Tn*917* isolated from *Streptococcus faecalis* known to transpose in *Bacillus* spp and *Listeria* spp.

For the purpose of the present invention a useful transposable element is one that mediate operon fusion and transcriptional fusion. Accordingly, such fusion-generating derivatives of a transposon which has lactic acid bacterial DNA molecules as their target, including derivatives of the above gram-positive transposons may be used in the present method. As an example, fusion-generating transposon derivatives may comprise a promoterless structural gene, the expression of which is readily detectable. Such a promoterless structural gene may e.g. be selected from a gene coding for a gene product conferring antibiotic resistance, a gene coding for a gene product complementing an auxotrophic deficiency or a gene coding for an enzyme having a readily detectable end product such as a product resulting in a colour reaction in an appropriate solid or liquid medium.

For example, the insertion of a promoterless *lacZ* gene into a plasmid comprising the transposon, in an orientation suitable for obtaining transposition-mediated fusions results in a plasmid vector that turns bacteria containing it, blue when grown on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) as a result of the expression of β-galactosidase. Transpositional insertions into the chromosome or into a plasmid, generated with such vectors produce white colonies, unless the insertions occur downstream of a functional promoter and in the right orientation to effect a transcriptional fusion. In this manner the promoterless gene serves as a promoter and/or operon probe gene. As another

example, a suitable fusion-generating transposon derivative may comprise the promoterless gene *cat-86* gene, the gene product of which mediate chloramphenicol resistance.

In the present context, an essential characteristic of a suitable transposable element is its ability to transpose with a high degree of randomness. Transposable elements vary greatly in target specificity, and their sites of insertion often exhibit little or no similarity to element sequences. Some elements may have from a few to hundreds of target sites in any gene, although no element has been found to insert completely randomly. Other elements are highly site specific, inserting into just a single chromosomal site. Yet other elements seem to insert quasi-randomly in some species, but prefer either particular regions of DNAs or certain regions of DNA molecules. For the purpose of the present invention a transposable element which is randomly or at least quasi-randomly integrated is preferred, the term "quasi-randomly" being defined herein as a degree of integration randomness in terms of the proportion of the total number of insertion events which is observed in a target DNA fragment of a known size relative to the proportion of insertions expected in this DNA fragment which is at the most 5, preferably at the most 4, more preferably at the most 3 and in particular at the most 2.5. In useful embodiments transposable elements which have a preference for chromosomal DNA may be preferred.

In certain preferred embodiments, a DNA molecule capable of replicating in a lactic acid bacterium and comprising a fusi-on-generating derivative of the Tn*917* transposon may be selected for the present method. Such derivatives include plasmids of the pTV series which include pTV32, pLTV1, pLTV3, pTV51, pTV52 and pTV53. Of these, pTV32 and pLTV1 may be particularly useful.

Furthermore, the DNA molecule as provided in step (i) of the present method comprises a detectable selective marker gene

allowing the selection of cells in which the DNA fragment has been introduced. In this connection, convenient marker genes include ones coding for gene products conferring resistance to antibiotics, e.g. resistance to macrolide antibiotics such as erythromycin and lincomycin; tetracycline, β-lactam antibiotics and chloramphenicol. As other examples, the marker gene may code for the complementation of auxotrophy in the host cell into which the DNA fragment is introduced or it may be a gene coding for an enzyme capable of generating a readily detectable end product such as e.g. the above-mentioned *lacZ* gene.

In a second step of the present method, the DNA molecule as defined above is introduced into a population of cells of a lactic acid bacterium. Such an introduction may be carried out in accordance with known techniques of introducing DNA into a host cells including transformation of protoplasted cells, transformation by electroporation or, if the DNA fragment is a conjugative element, by conjugation. The selected method should preferably result in a frequency of DNA introduction which is at least 10^4 recombinant cells per μg of DNA such as at least 5×10^4 per μg of DNA, e.g. at least 10^5 per μg of DNA.

In order to secure a high probability of obtaining integration of the transposable element into host cell DNA it is essential that the DNA molecule is one which is capable of replicating in the host cell. Accordingly, step (ii) may include a substep allowing the introduced replicon to replicate, followed by a procedure to study to what extent replication has occurred in the transformant or exconjugant. In the present context, a suitable extent of replication is considered to be a copy number which is in the range of 5 to 20 per cell. It is contemplated that a copy number substantially exceeding this range may render the curing of the replicons, which is an essential prerequisite for a subsequent transposition to occur, more difficult to achieve.

In a further substep, step (ii) comprises subjecting the transformant or exconjugant cells to conditions which allow transposition to occur. Transposition in non-lactic acid bacteria may be induced by one or more shifts in the environmental conditions of the cells. As an example hereof, the procedure for pTV-based Tn*917* mutagenesis in *B. subtilis* includes a step involving an antibiotic switch combined with a temperature upshift. Both Tn*917* *erm* gene expression and transposition are induced in *B. subtilis* by erythromycin (reference 57). In *B. subtilis*, the replication activity of pE194Ts-*rep* is blocked at temperatures above 37°C (reference 56). Consequently, curing for pTV plasmids, induction of and selection for transpositions are done by growing *B. subtilis* at temperatures exceeding 42°C in the presence of erythromycin.

During the experimentation leading to the present invention it was, however, found that the above procedure used in *B. subtilis* was not applicable to lactic acid bacteria such as the exemplified *Lactococcus lactis* ssp. *lactis* MG1614 and MG1363. However, it was surprisingly found that neither pTV32 nor pLTV1 could be extracted from the *Lactococcus* cells transformed with these plasmids when they were grown at 30°C in the presence of erythromycin. This indicated that transposition (integration) of the Tn*917* derivatives to the chromosome with a concomitant loss of plasmid had occurred under these conditions.

Accordingly, in one useful embodiment of the invention, step (ii) of the present method includes a substep where transposition of free transposable element-containing DNA molecules in transformed lactic acid bacteria is induced with a concomitant curing of such free molecules by growing the transformants at a temperature in the range of 20 to 35°C such as 30°C in the presence of an antibiotic to which the transposable element confers resistance.

In a subsequent step (iii) of the present method, integrant cells are cloned and subjected to a selection procedure to detect integrant cells wherein the promoterless gene of the transposable element is expressible. This selection procedure will depend on the type of the promoterless gene. When e.g. a promoterless *lacZ* gene is used, the selection may be carried out by plating the cloned integrants onto a medium containing a substance degradable by β-galactosidase with the development of a colour or, if an antibiotic resistance gene is used, the integrants may be selected on a medium supplemented with the corresponding antibiotic.

In step (iv) of the present method, a selected integrant expressing the promoterless structural gene is cloned and a lactic acid bacterial replicon region including a promoter being operably linked to the originally promoterless gene and possibly sequences regulating the function of the promoter is isolated from the cloned cells by the use of appropriate restriction enzymes. The resulting primary promoter-containing DNA sequences may have varying sizes depending on the location of restriction sites for the selected enzyme(s).

For further application of the isolated promoter-containing DNA sequences/fragments it may be advantageous to prepare subsequences of these primary sequences to obtain smaller fragments comprising the isolated promoter and possibly sequences regulating the function of the promoter. Whereas a primary promoter-containing fragment may e.g. have a size which is in the range of 40 to 600 kb, it is contemplated that a subsequence comprising the promoter and possibly other sequences required for its regulation may more appropriately have a size which is in the range of 50 to 10,000 base pairs.

In accordance with the present invention, the population of cells of a lactic acid bacterium into which the DNA fragment comprising the transposable element is introduced in the above-defined step (ii), are preferably selected from *Lactococcus* spp., *Streptococcus* spp., *Lactobacillus* spp., *Leuco-*

nostoc spp., *Pediococcus* spp., *Brevibacterium* spp., *Propionibacterium* spp. and *Bifidobacterium* spp. In one particularly preferred embodiment, the lactic acid bacterium is selected from *Lactoccus lactis* subspecies *lactis* such as the *Lactoccus lactis* ssp. *lactis* strains MG1614 and MG1363. During industrial use in food manufacturing of genetically improved lactic acid bacteria as defined herein it may be advantageous that the function of the bacteria is regulatable so that specific phenotypic traits of the lactic acid bacterial starter cultures may be turned on or switched off or the rate of expression of that trait is enhanced or reduced during specified periods of the manufacturing process including a maturation process. As an example it may be desirable in cheese manufacturing to use cultures which are not proteolytically or lipolytically active to a high degree during the curdling process but which are so during the maturation of the cheese.

Accordingly, the present method may, in one advantageous embodiment be a method wherein the promoter comprised in the DNA fragment being isolated and selected is a regulatable promoter. Such a method includes steps whereby the isolated promoter-containing sequences possibly including regulatory sequences are screened for mode of regulation. In the present context, a regulatable promoter may be regulatable by a factor selected from the pH and/or the content of arginine in the environment, the growth temperature, a temperature shift eliciting the expression of heat chock genes, the composition of the growth medium including the ionic strength/NaCl content, and the growth phase/growth rate of the lactic acid bacterium into which the promoter-comprising DNA molecule is introduced. One example of a promoter regulation mode is the phenomenon of stringent control by which is understood that the RNA synthesis of a cell is suspended in case the cell is starved for an essential nutrient such as an amino acid. Accordingly, a suitable regulatable promoter in accordance with the present invention may be one which is under stringent control.

An other example of a useful mode of regulating a promoter is to select a promoter that regulates a gene coding for an enzyme involved in the *de novo* synthesis of purine nucleotides from their precursors. By inserting into a lactic acid bacterium such a promoter which is regulated by being repressed in the presence of purine compounds, in front of a gene whose expression is to be regulated, this gene will only be expressed when the bacterium is growing in a medium not containing purine compound precursors. An example of such a regulated promoter is the lactococcal *purD* promoter as described hereinbelow.

As one example of screening for mode of promoter regulation, the isolated promoter may be screened for temperature/growth phase regulation by plating cells into which the promoter being operably linked to a gene coding for a gene product the expression of which is readily detectable, has been introduced by transposition, onto a suitable medium and incubating the plates at varying temperatures such as different temperatures within the range of 10 to 30°C and observing for temperature dependent gene expression. However, since the growth rate of the integrants cells will depend on the growth temperature it cannot be determined whether an observed apparently temperature-dependent expression is a result of a direct temperature regulation or the dependence is due to growth phase regulation.

Likewise, a possible pH and/or arginine dependent regulation of gene expression may be screened for by plating the above integrant cells onto media having different compositions which will result in varying pH values after growth of the integrant cell cultures. As an example the cells may be grown on GM17 medium where the final pH will be about 5 and on a modified GM17 medium having 1/5 of the normal glucose content and supplemented with 0.5% arginine. The pH in such a medium after growth of a culture of *Lactococcus lactis* integrant cells as defined above will be about 9. When expression of the gene under control of the isolated gene is only observed

at one of the two pH values, a pH and/or arginine dependent regulation is demonstrated.

Since one object of the present invention is to provide the means of constructing improved recombinant lactic acid bacteria by inserting promoter-containing sequences which result in enhanced expression of lactic acid bacterial gene(s) coding for desired gene products, it is part of the invention to screen promoter sequences for strength. This screening is carried out in accordance with methods which are known *per se*.

As it has been mentioned above, the present invention relates in a further aspect to a method of constructing a recombinant lactic acid bacterium containing a lactic acid bacterial gene coding for a desired gene product, the method comprising as a first step the isolation in accordance with the method as defined above, of a DNA sequence comprising a lactic acid bacterial promoter including where appropriate, additional regulatory sequences. The method comprises in a second step the insertion of the thus isolated DNA sequence into a lactic acid bacterium upstream of the lactic acid bacterial gene coding for the desired gene product so that the inserted promoter and possibly the above-defined regulatory sequences thereby becomes operably linked to the gene coding for a desired gene product.

The gene coding for a desired gene product may in accordance with the present invention be a homologous gene or it may be an inserted heterologous gene including a gene which is derived from a lactic acid bacterium. When the gene is an inserted gene it may be inserted on the same DNA sequence as that comprising the promoter sequence or it may be inserted on a separate DNA sequence.

In one useful embodiment, the insertion of the above isolated promoter-containing sequence may be on the chromosome of the lactic acid bacterium and in an other useful embodiment, the

sequence may be inserted extrachromosomally e.g. on a plasmid harboured by the bacterium. As it has been mentioned above, it may be advantageous to have the promoter-containing sequence integrated into the chromosome, since the sequence and the gene to which it is operably linked is hereby more stably contained as compared to a location on an extrachromosomal element. The insertion of the promoter-containing sequence is done according to gene technology methods which are known *per se* such as by insertion into a plasmid by conventional restriction and ligation procedures or integration into the chromosome by the use of transposons or bacteriophages or by conventional recombinational techniques.

In one interesting embodiment, the isolated promoter-containing sequence comprises a further sequence whereby the isolated promoter becomes regulated by a stochastic event. Such a regulation may e.g. be useful in lactic acid cultures for which it is advantageous to have a gradually decreasing activity of the gene under control of the inserted promoter-containing sequence. Such further sequences may e.g. be sequences which result in a recombinational excision of the promoter or of genes coding for substances which are positively needed for the promoter function.

A stochastic regulation of the promoter function may also be in the form of recombinational excision of a regulatory sequence inhibiting the function of the promoter whereby a gradually increasing promoter activity is obtained at the recombinant cell population level.

As it is mentioned above, the present invention provides a further method of constructing a recombinant lactic acid bacterium which bacterium comprises a gene coding for a desired gene product, the expression of which is altered as compared to expression of the gene when it is operably linked to its native promoter. In this method a DNA molecule as defined above and comprising a transposable element with a promoter probe gene is utilized to identify a site/sites in a

lactic acid bacterial replicon (chromosome or plasmid) in which the transposable element is integratable and where the promoterless probe gene becomes operably linked to a promoter sequence present in the replicon and subsequently, inserting in a non-integrant lactic acid bacterial cell at that or these sites or at a site/sites which are functionally equivalent thereto, a gene coding for a desired gene product, whereby this gene becomes operably linked to the identified promoter sequence.

Whereas the transposable element will become inserted between two base pairs, it will be understood that a gene coding for a desired gene product may, besides being inserted between those two base pairs also be inserted at a neighbouring site which is located at a distance from that specific insertion (integration) site which will still allow the identified promoter sequence to control transcription of the inserted gene. In the present context, such neighbouring sites are referred to as functionally equivalent sites. It is contemplated that the distance from the specific transposon integration site where such functionally equivalent sites may be found is within the range of 1 to 2000 base pairs.

In accordance with the invention, the gene coding for a desired gene product which is inserted into the above-defined site may be a homologous or a heterologous gene including a gene derived from a lactic acid bacterium.

The present invention provides in a further aspect a recombinant lactic acid bacterium comprising a gene coding for a desired gene product and operably linked thereto a lactic acid bacterial promoter not natively associated with the gene, the presence of said promoter resulting in the expression of the gene being altered as compared to the expression of the gene when operably linked to its native promoter.

As used herein, the term "altered expression" is used to indicate that the regulation of the expression of the gene

quantitatively or qualitatively different from the regulation of the gene when operably linked to its native promoter. A quantitatively different expression may be recognizable as an increased level of expression of the gene products such as at least a 10% increased expression. It may e.g. be advantageous that the expression is increased by at least 25% such as at least 50%. In certain embodiments it may be advantageous to provide recombinant lactic acid bacteria in which expression of the gene coding for a desired gene product is less than that of the gene when under control of its native promoter. Accordingly, a useful recombinant bacterium may have an expression the level of which is at least 10% reduced, preferably at least 25% or more preferably at least 50% reduced.

Qualitatively, the expression of a gene coding for a desired gene product, the native promoter of which is a constitutive promoter may be altered by operably linking it to a regulatable promoter or the expression of a gene having a native regulatable promoter may be altered by linking it to a constitutive promoter. In further embodiments, the expression of a gene having a native regulatable promoter may be qualitatively altered by linking it to a regulatable promoter having a different mode of regulation.

In one useful embodiment, the present invention provides the recombinant lactic acid bacterium as one comprising an inserted lactic acid promoter-comprising DNA sequence as defined above, the lactic acid bacterial promoter being operably linked to a gene coding for a desired gene product. The gene coding for a desired gene product may in accordance with the invention be a chromosomal gene or an extrachromosomally located gene.

In certain preferred embodiments, the above gene coding for a desired gene product may be a native gene which in the present context is defined as a homologous gene which is in its natural position on a chromosome or on a plasmid naturally occurring in a particular lactic acid bacterium or it may be

a homologous gene which is isolated from its natural position and reinserted into the same lactic acid bacterial strain, but in an other position. In still other useful embodiments, the gene coding for a desired gene product is a heterologous gene isolated from a non-lactic acid bacterial species or from an other lactic acid bacterial species.

Although it may in certain embodiments be preferred that the inserted promoter is a regulatable promoter, it may also in other useful embodiments be advantageous to provide a recombinant lactic acid bacterium wherein the inserted promoter is a constitutive promoter. When the selected promoter to be inserted is a regulatable promoter, the mode of regulation may be selected from the factors as defined hereinbefore, including regulation by a stochastic event.

It may be advantageous to provide the lactic acid bacterium according to the present invention as one in which an inserted DNA sequence comprising a lactic acid bacterial promoter is inserted into a plasmid. In certain preferred embodiments such a plasmid is one which further comprises a gene coding for a desired gene product as defined herein, a lactic acid bacterial replicon which is functional in a lactic acid bacterium, an insertion site allowing the DNA sequence to be inserted so that the gene coding for the desired gene product is operably linked to the promoter, whereby the gene can be transcribed when the plasmid is present in a lactic acid bacterium.

The promoter inserted into the plasmid may preferably be a promoter which is regulatable as it is described herein.

In this context, a suitable lactic acid bacterium is one harbouring the plasmid pAK80 which is described in the following or a derivative hereof including pAK80:SB, pAK80:143, pAK80:162, pAK80:163, pAK80:170, pAK80:224 and pAK80:242.

In an interesting embodiment, the lactic acid bacterium to be recombined in accordance with the present invention may carry the gene coding for a desired gene product on a plasmid having a conditional replication behaviour so that the plasmid copy number under certain conditions is substantially increased, e.g. to several hundreds or thousands. Plasmids having such a replication behaviour is also designated run-away plasmids.

A recombinant lactic acid bacterium as defined herein may be one which is selected from *Lactococcus* spp. including *Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *diacetylactis* and *Lactococcus lactis* ssp. *cremoris*, *Streptococcus* spp. including *Streptococcus salivarius* ssp. *thermophilus*, *Lactobacillus* spp. including *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus delbrückii* ssp. *bulgaricus*, *Lactobacillus helveticus*, *Leuconostoc* spp. including *Leuconostoc oenos*, *Pediococcus* spp., *Brevibacterium* spp., *Propionibacterium* spp. and *Bifidobacterium* spp. including *Bifidobacterium bifidum*.

In preferred embodiments, the recombinant lactic acid bacterium is one in which the inserted promoter-containing sequence as defined herein is derived from *Lactococcus* spp. such as from *Lactoccus lactis* subspecies *lactis*., *Streptococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* spp., *Brevibacterium* spp., *Propionibacterium* spp. and *Bifidobacterium* spp. In certain specific embodiments, the inserted promoter may be isolated from *Lactoccus lactis* subspecies *lactis* strains MG1614, MG1363 or CHCC285 (Chr. Hansens Laboratorium A/S). Interesting promoters are tRNA and rRNA promoters including the PI and PII promoters and the purD promoter from *Lactoccus lactis* subspecies *lactis* as described in the following. Particularly interesting promoters are strong promoters such as tRNA or rRNA promoters which comprise the conserved sequence (motif) AGTT.

The present recombinant lactic acid bacterium is preferably one in which the gene coding for a desired gene product is selected from a gene coding for a lipase, a gene coding for a nuclease, a gene coding for a peptidase such as an aminopeptidase, a gene coding for a protease, a gene coding for a gene product involved in carbohydrate metabolism, a gene coding for a gene product involved in citrate metabolism, a gene coding for a gene product involved in bacteriophage resistance, a gene coding for a lytic enzyme such as lysozyme or a phage lytic enzyme and a gene coding for a bacteriocin including nisin. In an interesting aspect, the gene coding for a desired gene product may be one the gene product of which confer resistance to a bacteriocin such as nisin, or pediocin.

The above genes coding for a desired gene product may be genes derived from a lactic acid bacterium or they may suitably be genes derived from a non-lactic acid bacterial microbial species or from a eucaryotic cell including plant cells and human or animal cells. As one example of a useful gene derived from a eucaryotic cell may be mentioned plasminogen.

In one specific preferred embodiment of the invention the gene is selected from the *lacL* gene of a *Leuconostoc* spp., the *lacM* gene of a *Leuconostoc* spp. and a *Lactococcus lactis* ssp. *lactis* gene coding for a peptidase such as a lysine aminopeptidase.

In accordance with the present invention the recombinant lactic acid bacterium as defined herein may suitably be one in which a gene coding for a desired gene product is inserted at a site in a replicon where it is under the control of a promoter present in the replicon, which site is identifiable by the insertion of a promoterless structural gene by means of a transposable element comprising the promoterless structural gene whereby the originally promoterless gene becomes expressible by being operably linked to the promoter present in said replicon, the insertion of the gene at said site

having resulted in said gene becoming operably linked to the promoter being present in the replicon.

It will be understood that the site at which the gene coding for a desired gene product may be inserted is not limited to the specific site between two base pairs as identified by the insertion of the transposable element, but may be any site within a distance from this specific site which may still allow the lactic acid bacterial promoter to which the promoterless gene of the transposable element may become operably linked, to control the expression of the inserted gene. Insertion sites which are in such a distance from the specifically identified site may in the present context be referred to as functionally equivalent insertion sites.

There may also in accordance with the present invention be provided a recombinant lactic acid bacterium into which has been inserted a promoter-comprising sequence as defined above as well as a gene coding for a desired gene product also as defined above.

As mentioned above, the present invention provides in a still further aspect an isolated DNA fragment comprising (i) a regulatable lactic acid bacterial promoter which is functional in a lactic acid bacterium and operably linked thereto (ii) a gene coding for a desired gene product, said promoter being one which is not naturally associated with the gene and which confers to the gene coding for a desired gene product an altered expression as defined hereinbefore.

Such a DNA fragment is isolated in accordance with the method as described herein. In one useful embodiment the DNA fragment is one which further comprises at least one transcription terminator. The present DNA fragment is preferably a fragment having a size which is in the range of 100 to 10000 base pairs such as a size which is in the range of 200 to 5000 base pairs. In accordance with the invention, the DNA fragment may also be one which further comprises sequences

coding for gene products involved in the regulation of the promoter.

In useful embodiments, the DNA fragment is one in which the gene coding for a desired gene product is selected from a gene coding for a lipase, a gene coding for a peptidase, a gene coding for a protease, a gene coding for a gene product involved in carbohydrate metabolism, a gene coding for a gene product involved in citrate metabolism, a gene coding for a gene product involved in bacteriophage resistance, a gene coding for a lytic enzyme and a gene coding for a bacteriocin. The gene may also be one which codes for a gene product conferring resistance to an antibiotic or a bacteriocin such as e.g. nisin or pediocin.

The DNA fragment as defined above may comprise a gene coding for a desired gene product which is a homologous or a heterologous gene including a gene derived from a lactic acid bacterium. Accordingly, the gene may in certain preferred embodiments be one which is selected from the *lacL* gene of a *Leuconostoc* spp., the *lacM* gene of a *Leuconostoc* spp. and a *Lactococcus lactis* ssp. *lactis* gene coding for a lysine aminopeptidase.

The lactic acid bacterial promoter comprised in the DNA fragment may be isolated from any lactic acid bacterial species as mentioned herein and may be a constitutive or regulatable promoter as also defined above. In specific embodiments of the invention the promoter is selected from the regulatable promoter contained in the *Lactococcus lactis* ssp. *lactis* MG1363 integrant clone P139-170 deposited under the accession number DSM 7360 and the promoter contained in the *Lactococcus lactis* ssp. *lactis* MG1614 integrant clone 63b deposited under the accession number DSM 7361.

The recombinant bacterium may in accordance with the invention be one in which the inserted DNA sequence comprising a regulatable lactic acid bacterial promoter is inserted into a

vector comprising a promoterless gene coding for a desired gene product, a theta-replicating lactic acid bacterial replicon which is functional in the bacterium, an insertion site allowing the DNA sequence to be inserted so that the gene coding for the desired gene product is operably linked to the promoter, whereby the gene is transcribed. In one embodiment such a bacterium may as the vector into which the inserted DNA sequence is inserted comprise the plasmid pAK80.

The recombinant lactic acid bacterium as provided herein may be useful in starter cultures for the manufacturing of food products including dairy products, meat products and vegetable products and in the preservation of animal feed. In the latter context, the present recombinant bacteria are particularly interesting as inoculants in field crops which are to be ensiled. When the bacteria are to be used for these purposes they may conveniently be provided in the form of dried or frozen bacterial concentrates e.g. containing 10^{10} to 10^{12} colony forming units (CFUs) per g of concentrate.

An interesting use of a recombinant lactic acid bacterium as defined herein is in the manufacturing of a probiotically active composition. The term "probiotically active" indicates that the bacteria selected for this purpose have characteristics which enables them to colonize in the gastrointestinal tract and hereby exert a positive regulatory effect on the microbial flora in this habitat. Such effect may be recognizable as an improved food or feed conversion in human or animals to which the bacteria are administered, or as an increased resistance against invading pathogenic microorganisms.

Furthermore, it is contemplated that the present recombinant lactic acid bacteria may be useful in the preparation of recombinant vaccine strains in which one or more genes coding for antigenic determinants are inserted.

The recombinant plasmid according to the present invention is preferably one in which the lactic acid bacterial promoter is a promoter which is regulatable in a manner such as it has been defined hereinbefore. In this context useful plasmids may be selected from the plasmid pAK80 or a derivative hereof including pAK80:SB, pAK80:143, pAK80:162, pAK80:163, pAK80:170, pAK80:224 and pAK80:242.

The invention is further illustrated in the following Examples and Figures, where:

Figure 1 is a map of pTV32 in which the following abbreviations indicate restriction enzyme sites: SalI, EcoRI, PstI, XbaI, KnpI and SmaI, Tn917 indicates the transposon part, erm indicates the gene coding for erythromycin resistance, bla the gene coding for β-lactamase, ColEI rep the origin of replication of the ColEI plasmid, cat indicate the gene coding for chloramphenicol acetyltransferase mediating resistance to chloramphenicol, lacZ the promoterless β-galactosidase gene or *E. coli*, tet indicates the gene coding for tetracycline resistance and pE194 Ts rep indicates the temperature sensitive origin of replication derived from plasmid pE194,

Figure 2 is a map of pLTV1 (abbreviations, cf. the legend to Figure 1),

Figure 3 illustrates Southern hybridization analysis of 12 independent *L. lactis* ssp. *lactis* TV32 integrants. DNA from integrants, indicated on top of each lane, was digested with EcoRI, electrophoresed through an agarose gel, transferred to a nylon membrane and hybridized with A: ^{32}P labelled pLTV1. B: ^{32}P labelled pE194 replicon-specific probe. Size markers are given in kilobase pairs,

Figure 4 shows pulsed-field gel electrophoresis (PFGE) of SmaI-digested DNA from *L. lactis* ssp. *lactis* TV32 integrants. Integrant numbers are indicated on top of the lanes. A:

lambda ladder (Promega, Madison, USA) starting from the bottom with 48.5 kb, 97.0 kb, 145.5 kb etc. B: delta 39 lambda ladder (Promega) starting from the bottom with 39.0 kb, 78.0 kb, 117 kb etc. M is SmaI-digested DNA from *L. lactis* ssp. *lactis* MG1614,

Figure 5 illustrates pulsed-field gel electrophoresis (PFGE) of 19 clones (E1-E19) picked from a culture of *Lactococcus lactis* ssp. *lactis* MG1614 comprising a dominant TV32 integrant. Lanes indicated by A, B and M are as indicated above for Figure 5. The digestion of clone E5 resulted in fragments which could not be visualized as discrete bands,

Figure 6 illustrates pulsed-field gel electrophoresis (PFGE) of 18 clones (K1-K2, K4-K14, K16-K20) picked randomly from a pooled culture of *Lactococcus lactis* ssp. *lactis* MG1614 TV32 integrants. Lanes indicated by A, B and M are as indicated above for Figure 5,

Figure 7 shows the streak pattern for investigation of regulated *lacZ* expression in promoter fusion clone collection no. 1. Each clone was streaked onto a plate containing 1 μg/ml erythromycin and 320 μg/ml of X-gal in a straight line of about 0.5 cm,

Figure 8 illustrates the construction of pAK67.7 as described in Example 6. P represents the β-galactosidase promoter of *Leuconostoc mesenteroides* subsp. *cremoris*, and rbs the ribosome binding site. The sites of homology to the primers lac-1 and lac-2 are indicated by small arrows. The ribosome binding site is also present in pAK67.7,

Figure 9 illustrates the growth and β-galactosidase activity of the LTV1 integrant 170 grown at pH 5.5 and 7.0,

Figure 10 illustrates the growth and β-galactosidase activity of the LTV1 integrant SB grown at pH 5.5 and 7.0,

Figures 11 illustrates a DNA fragment from *Lactococcus lactis* subsp. *lactis* strain CHCC285 containing seven tRNA genes and a 5S rRNA gene arranged in a single operon including two promoters and two putative transcription terminators,

Figure 12 shows the gene organization and nucleotide sequence of *trnA*. The deduced amino acid sequence of *'tma* is shown in one-letter code below, the stop codon indicated by an asterisk. Putative -35 and -10 promoter sequences (PI, PII), a conserved motif in the -44 region and a conserved sequence that might be involved in stringent control (Chiaruttini & Milet, 1993; Ogasawara et al., 1983) are double underlined. The coding regions of the tRNA genes and *rrfU* are underlined. Putative transcription terminators are indicated by arrows above the sequence. The location of restriction enzyme sites for *Sca*I and *Spe*I, used for the cloning and promoter cloning, is shown above the sequence,

Figure 13 shows a comparison of tRNA and rRNA promoter sequences from *Lactococcus lactis* and *Lactococcus cremoris*. The conserved -44 region, -35 region, a doublet TG (cf. reference 19), -10 and a conserved sequence suggested to be involved in control of expression during the stringent response of *Bacillus subtilis* (Ogasawara et al., 1983) are underlined. A: PI of *trnA* ; B: PII of *trnA*; C: P21 from a *Lactococcus cremoris* $tRNA^{leu}$ gene (van der Vossen et al., 1987; this study); D: P2 from *Lactococcus lactis* (Koivula et al., 1991); E: promoter region in front of a *Lactococcus lactis* ochre suppressor gene (F. Dickely & E. Bech Hansen, personal communication); F: P10 from a *Lactococcus lactis* $tRNA^{arg}$ gene (Koivula et al., 1991; this study); G: promoter of a *Lactococcus lactis* rRNA operon (Chiaruttini & Milet, 1993); H: P2 from a *Lactococcus lactis* rRNA operon (Beresford & Condon, 1993); I: putative promoter in front of a *Lactococcus lactis* amber suppressor gene (E. Johansen, unpublished results); J: P21 from *Lactococcus lactis* (Koivula et al., 1991). Con., shows identical nucleotides in the aligned sequences A to H,

Figure 14 is a 846 bp DNA fragment from *Lactococcus lactis* containing the entire *purD* promoter region as well as an adjacent promoter initiating transcription in the opposite direction,

Figure 15 illustrates the OD_{600} and the β-galactosidase activity versus time during fermenter growth of pSMA344/MG-1363 in liquid medium under controlled conditions,

Figure 16 is a restriction map of a 9.7 kb lactococcal *Eco*RI-*Cla*I fragment from p170 and of deletion derivatives,

Figure 17 is a restriction map of a 4.0 kb lactococcal *Nde*I-*Cla*I fragment of p170 and of deletion derivatives, and

Figure 18 illustrates the Campbell-like integration of a non-replicating plasmid into the lactic acid bacterial chromosome where P represents a promoter, Erm represent an erythromycin resistance gene, reporter gene is the β-galactosidase gene from *Leuconostoc mesenteroides* and the *E. coli* replicon is the pACYC replicon from pVA891 and where black areas illustrate the region of DNA homology between the plasmid and the chromosome and arrows indicate the direction of transcription from the promoter P.

EXAMPLE 1

Transformation of *Lactococcus lactis* ssp. *lactis* MG1614 with pTV32 and pLTV1 and demonstration of replication of these plasmids

Several vectors (pTV plasmids) containing derivatives of the transposon Tn*917* from the lactic acid bacterial species *Streptococcus faecalis* have been constructed for use in *Bacillus subtilis* and other gram-positive bacteria (references 10, 55 and 57). Two derivatives of the pTV plasmid

series, pTV32 (reference 57) and pLTV1 (reference 55) were selected for this and the following experiments.

pTV32 (15.6 kb) and pLTV1 (20.6 kb) contain (i) a temperature sensitive replicon (pE194Ts-*rep*) from the plasmid E194, (ii) on the replicon part of the plasmid, a *cat* gene (pTV32) which confers chloramphenicol resistance (Cm^r) or tetracycline resistance (Tc^r) gene (pLTV1), (iii) Tn*917* harbouring an *erm* gene which confers erythromycin resistance (Em^r), and (iv) a promoterless *E. coli lacZ* gene with a ribosomal binding site from *Bacillus subtilis* inserted in non-essential Tn*917* DNA at the *erm*-proximal end (Figures 1 and 2). pTV32 and pLTV1 were isolated from *E. coli* PY1173 and *Bacillus subtilis* PY258, respectively. These strains were obtained from P. Youngman, University of Pennsylvania.

Lactococcus lactis ssp. *lactis* MG1614 which is a prophage-free, plasmid-free, streptomycin- and rifampicin resistant derivative of strain NCDO 712 was transformed with pTV32 or pLTV1 using the electroporation method described by Holo and Ness (reference 20) and primary transformants were selected by plating onto M17 medium (Sigma Chemical Co.) containing 0.5% glucose (GM17 medium) supplemented with 0.5 M sucrose, 2mM $CaCl_2$ (SGM17,Ca medium) and the appropriate selective antibiotic (erythromycin or chloramphenicol) and incubated at 30°C. The antibiotics were purchased from Sigma and were used at the following concentrations: erythromycin, 1.0 $\mu g\ ml^{-1}$; chloramphenicol, 5.0 $\mu g\ ml^{-1}$.

With either plasmid, the transformation efficiencies were 10^4 to 5 x 10^4 transformants per μg of DNA when selecting for Cm^r or Em^r.

Selected primary transformant colonies were transferred to GM17 liquid medium supplemented with 5.0 $\mu g\ ml^{-1}$ of chloramphenicol and the transformant cells were grown up till a number of generations being in the range of 10 to 50. Plasmid DNA was subsequently extracted from these transformants by

performing an alkaline lysis of the cells substantially in accordance with the method described by Birnboim et al. (reference 6) with modifications as indicated in the following. Cells were grown exponentially to an A_{600} of 0.3, and 5 ml cultures were harvested by centrifugation at 4,000 x g. Pellets were washed in TS buffer (25% sucrose, 50 mM Tris hydrochloride, pH 8.0), resuspended in 0.25 ml S1 solution (5 mM EDTA, 50 mM NaCl, 25% sucrose, 50 mM Tris hydrochloride, pH 8.0) with 10 mg/ml lysozyme and incubated at 30°C for 30 min. 0.5 ml S2 solution (0.2 M NaOH, 1% SDS) was gently added and the suspension kept on ice for 5 min. Subsequently 0.4 ml 3 M sodium acetate pH 4.8 was added and the suspension kept on ice for 5 min. Following centrifugation of the suspension at 10,000 x g, plasmids were extracted from the supernatant in accordance with the method described by Birnboim et al (reference 6).

A portion of the thus extracted plasmid DNA and plasmids pTV32 and pLTV1 isolated from *E. coli* PY1173 and *Bacillus subtilis* PY258, respectively were subjected to a treatment under standard conditions with the restriction enzymes *Eco*RI, *Sal*I and *Hind*III. Undigested extracted plasmid DNA isolated from *Lactococcus lactis* ssp. *lactis* MG1614 and from *E. coli* PY1173 and *Bacillus subtilis* PY258 as well as the restriction enzyme treated plasmid DNA were then subjected to an agarose gel electrophoresis analysis and it was found that the sizes of pTV32 and pLTV1 extracted from the transformed *Lactococcus lactis* ssp. *lactis* MG1614 as well as the restriction enzyme sites *Eco*RI, *Sal*I and *Hind* III were retained as compared to with the original plasmids. By assuming that the level of recovery in the above plasmid preparation procedure was 100%, the average copy number of both the plasmids in the transformed *Lactococcus lactis* ssp. *lactis* MG1614 was estimated to be 6 to 12 copies per cell by performing a comparison on agarose gels with a standard of phage lambda DNA of known concentration digested with *Hind*III. Accordingly, it could be concluded from this experiment that lactic acid bacteria may be transformed with pTV32 and pLTV1 at a high efficiency and

that these plasmids are capable of replicating in a lactic acid bacterium.

EXAMPLE 2

Induction of Tn*917* transposition in *L. lactis* ssp. *lactis* and curing for pTV-plasmids

Lactococcus lactis ssp. *lactis* MC1614 ceases to grow in M17 broth (Sigma Chemical Co.) containing 0.5 glucose at temperatures exceeding 37°C. Since pTV32 or pLTV1 could be extracted from *L. lactis* ssp. *lactis* MG1614 transformed with these plasmids and grown at 37°C under selection for Cm^r, the temperature curing procedure developed for *B. subtilis* could not be used in the *Lactococcus* strain.

However, it was demonstrated that neither pTV32 DNA nor pLTV1 DNA could be extracted from *Lactococcus* transformants grown at 30°C with selection for Em^r. This indicated transposition (integration) of Tn*917* to the chromosome with concomitant loss of plasmid.

Production of independent *Lactococcus lactis* ssp. *lactis* Tn*917* integrants from individual cultures were carried out according to the following procedure:

Primary transformed cells prepared as described in Example 1 were plated on SGM17,Ca agar containing erythromycin and incubated at 30°C for about 40 hours. 12 single colonies were subcultured twice in M17 broth medium selecting for Em^r. In order to obtain single colonies each culture was streaked on GM17 agar containing erythromycin and a single colony from each culture was restreaked once. All incubations were done at 30°C.

To verify that these assumed independent integrants had lost the plasmids as free molecules and had Tn*917* inserted in the

chromosome, a Southern hybridization was carried out on DNA from the 12 independent Em^r MG1614 clones initially transformed with pTV32 and subcultured twice in liquid medium selecting for Em^r. From the isolates, the total DNA content was extracted from 100 ml cultures by harvesting the cells by centrifugation at 7000 rpm for 10 minutes. The cells were washed in TE buffer (10 mM Tris hydrochloride, 1 mM EDTA pH 7.5) and harvested. The pellets were frozen at -20°C and subsequently dissolved in 3 ml STET buffer (8 w/v% sucrose, 5 v/v% Triton X-100, 50 mM EDTA [pH 8.0], 50 mM Tris hydrochloride [pH 8.0]. 750 µl lysozyme (10 mg/ml) was added and the solution incubated at 37°C for 1 hour. 750 µl of 10% SDS was added and incubation continued at 37°C for 1/2 hour followed by incubation at 65°C for 1/2 hour. Two ml of TE buffer was added and the aqueous solution extracted three times with 5 ml phenol:chloroform (1:1). To the suspension 1/10 volume of 5 M NaCl and 1 volume of isopropanol was added. The solution was mixed very carefully until DNA precipitated as long white threads. The DNA was wound on an inoculation needle and transferred to Eppendorf tubes and washed 3 times in 70% ethanol. The DNA was dissolved in 500 µl of TE buffer.

1 µg of the thus prepared DNA from each isolate was digested with *Eco*RI and separated by electrophoresis through 1.0% agarose gels and transferred to Hybond-N membranes (Amersham, UK) and subjected to hybridization using two ^{32}P-labelled DNA probes, viz pLTV1 and a 4 kb *Eco*RI fragment of pLTV1 containing the pE194 replicon. The 4 kb fragment was isolated from agarose gels by electroelution into dialysis bags. The probes were nick translated with [α-^{32}P]dCTP (Amersham, UK). The restriction enzyme digestion, electrophoresis, DNA transfer, nick translations and hybridizations were done as described by Maniatis at al. (reference 34).

The integrant clones hybridized with the ^{32}P-labelled pLTV1 and/or the pTV replicon-specific probe as illustrated in Figure 3. pTV32 is 15.6 kb and has a unique *Eco*RI site which

is located in the replicon part of the plasmid. The Tn*917* part of pTV32 is 8 kb. From 8 out of the 12 TV32 integrants a single signal was detected with pLTV1 as the probe (Figure 3A) whereas no signal was seen with the pTV-replicon specific probe (Figure 3B). These 8 integrants were Em^r and Cm^s as would be expected if TV32 had transposed to the chromosome and pTV32 was lost. From the remaining four integrants (number 27, 33, 36 and 39) two signals were detected with pLTV1 as the probe (Figure 3A). The same two bands hybridized with the replicon specific probe and no signal of the size expected for freely replicating pTV32 was observed (Figure 3B). Accordingly, these four strains had DNA from the replicon part of pTV32 integrated into the chromosome together with the transposon TV32. In each of the four integrants, the DNA from the replicon part included the *cat* gene, since all four were Cm^r.

EXAMPLE 3

Demonstration of quasi-randomness of Tn*917* insertion into the *Lactococcus lactis* ssp. *lactis* MG1614 chromosome

In order for Tn*917* to be used as an efficient mutagenesis tool in *L. lactis* ssp. *lactis*, insertions of the transposon should be random. An analysis of transposition randomness was carried out by determination of the physical location of TV32 on chromosomal *Sma*I fragments of 61 independent MG1614 TV32 integrants which were prepared according to the method as described in Example 2. The preparation and *Sma*I *in situ* restriction enzyme digestion of genomic DNA was done as described by Tanskanen et al. (reference 52). Of these integrants, 10 expressed β-galactosidase as shown by plating on GM agar supplemented with 160 μg/ml of X-gal.

The *Sma*I restriction fragments were separated by pulsed-field gel electrophoresis (PFGE) using a model CHEF-DR II apparatus (Bio Rad Laboratories, Richmond, California). The gels were

1.5% agarose gels in 0.5 x TBE (1 x TBE in 89 mM boric acid, 2 mM EDTA and 89 mM Tris borate [pH 8.3]. The electrophoresis parameters were as follows: 175V for 20 hours at 14°C with ramped pulse times for 1 to 70 seconds. The gels were stained with an ethidium bromide solution (1 mg/ml) in 0.5 x TBE for 30 minutes, destained for 4 hours in 0.5 x TBE and photographed using a UV transilluminator.

The MG1614 chromosome digested with *Sma*I generated the following ten fragments larger than 45 kb (Fig. 4, lane 3): 600, 310, 280, 200, 175, 175, 140, 120, 105 and 65 kb. TV32 contains a unique *Sma*I site. The insertion of TV32 into any of the ten large *Sma*I fragments was therefore detectable on pulsed-field gel electrophoresis (PFGE) gels unless the insertion was located close to the fragment end.

The TV32 locations on the *Sma*I fragments of the 61 integrants are given in Table 1.

Table 1. Random *Lactococcus lactis* ssp. *lactis* TV32 integrants divided into groups on the basis of the physical location of TV32 on chromosomal SmaI fragments

Group	TV32 target: chromosomal *Sma*I fragment (kb)	Fragment lengths (kb) of *Sma*I-digested target fragments with inserted TV32[a]	Group members (integrant No.)[b]
1	600	540 + 70 (= 610)	70b
2	600	535 + 75 (= 610)	21, 44
3	600	530 + 80 (= 610)	4, 27, 31
4	600	525 + 85 (= 610)	39, 49
5	600	505 + 105 (= 610)	22
6	600	485 + 125 (= 610)	3, 30
7	600	470 + 140 (= 610)	34
8	600	460 + 145 (= 605)	35, 40, 54
9	600	450 + 160 (= 610)	41, 42

10	600	445 + 165 (= 610)	61b, 62b, 68b
11	600	440 + 170 (= 615)	33
12	600	405 + 200 (= 605)	1, 20
13	600	390 + 205 (= 605)	6
14	600	380 + 225 (= 605)	12
15	600	375 + 230 (= 605)	10, 43
16	600	360 + 235 (= 595)	11, 23, 65b
17	600	355 + 240 (= 595)	50
18	600	350 + 245 (= 595)	16, 64b
19	600	325 + 280 (= 605)	66b
20	600	310 + 300 (= 610)	25, 38
21	310	245 + 65 (= 310)	45
22	310	185 + 135 (= 320)	60
23	310	180 + 140 (= 320)	8
24	200	150 + x	19[c]
	600	420 + 210 (= 630)	
25	175	155 + x	29
26	175	140 + x	47
27	175	105 + 75 (= 180)	24
28	140	115 + x	13, 15
29	140	110 + x	2, 26, 63b
30	140	105 + x	18, 32, 51, 59
31	140	100 + x	14
32	140	90 + x	7
33	140	85 + x	5, 36
34	120	115 + x	69b
35	120	110 + x	48
36	120	105 + x	55
37	120	90 + x	67b
38	ND[d]		46

[a] x indicates a fragment that could not be detected on PFGE gels.

[b] Clones whose designations end with b are blue on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

[c] Double integrant.

[d] ND, not determined.

Based on the physical location of TV32 on the *Sma*I fragments, the 61 integrants could be divided into 38 groups. Fig. 4 shows PFGE of integrants representing each of the groups listed in Table 1. One group (No. 30) contained four integrants, five groups (Nos. 3, 8, 10, 16 and 29) contained three integrants and ten groups (Nos. 2, 4, 6, 9, 12, 15, 18, 120, 28 and 33) contained two integrants. However, members of the same integrant group do not necessarily carry the TV32 at the same position on the fragment. Insertions located symmetrically on a fragment are indistinguishable on PFGE gels and the limit of resolution varies from two to ten kb depending on the fragment length.

The 600, 310, 200, 175, 140 and 120 kb chromosomal *Sma*I fragments had all been targeted by TV32 (Table 1 and Fig. 4). Apparently none of the integrants carried insertions in the 280, 105 and 65 kb fragments. However, it could not be established from the PFGE data if the TV32 in integrant 46 resided at the end of a fragment larger than 45 kb or in any position on a fragment smaller than 65 kb in size. Integrant 19 contained a double insertion (Fig. 4). Two TV32 copies are carried on the 200 kb and the 600 kb fragments, respectively. These double insertions make the total number of insertion events in this study 62.

The 62 TV32 insertions in the *Lactococcus lactis* ssp. *lactis* chromosome were not evenly distributed along the chromosome. This was revealed by a chi-square analysis whereby it was tested whether the probability of insertion into a *Sma*I fragment was dependent only on the length of the fragment (Table 2).

Table 2 gives the number of integrants obtained in each fragment, together with the expected number of integrants assuming that the probability of integration into a fragment is dependent only on the length of the fragment. A chi-square test was used to test this assumption. The chi-square test

showed ($P < 0.005$) that the insertions obtained were not absolutely randomly distributed on the chromosome. The major contribution to this unevenness came from a 2.5-fold overrepresentation of insertions into the 600 kb fragment and an absence of insertions into 280 kb fragment. The 37 insertions into the 600 kb fragment were located at least 21 different positions with no more than 3 insertions at the same position. These results indicate that the above overrepresentation cannot not be due to a single dominating hot spot. The 280 kb fragment is not totally refractory to TV32 insertions, since such integrants were obtained in parallel experiments. Accordingly, in the present context the TV32 insertion distribution pattern as obtained in *Lactococcus lactis* ssp. *lactis* strain MG1614 is designated as "quasi-random".

The following factors may have contributed to the observed uneven distribution of insertions: (1) fragments near the chromosomal origin of replication have higher copy numbers than fragments near the terminus; (2) essential genes may have been unevenly distributed; and (3) Tn*917* might become preferentially inserted into regions with particular features.

Table 2. Distribution of TV32 on chromosomal *Lactococcus lactis* ssp. *lactis* *Sma*I fragments

TV32 target: chromosomal *Sma*I fragment (kb)[a]	No. of insertions observed[b]	No. of insertions expected[c]	No. of insertions observed/ No. of insertions expected	Chi-square test[d]
600	37	14.9	2.5	32.8
175[e]	3	8.7	0.3	3.7
310	3	7.7	0.4	2.9
280	0	6.9	0.0	6.9
200	1	5.0	0.2	3.2
140	13	3.5	3.7	
120	4	3.0	1.3	
105	0	2.6	0.0	0.0
65	0	1.6	0.0	
<45	1	8.2	0.1	

a) The total for the chromosomal *Sma*I fragment sizes was 2500 kb

b) The total number of insertions observed was 62

c) The probability of insertion was assumed to equal fragment size relative to chromosome size. The total number of insertions expected was 62.1.

d) Values were calculated as follows: (number of insertions observed - number of insertions expected)^2/number of insertions expected. The chi-square test requires the expected number for each class to exceed or equal 5; therefore, insertions in fragments smaller than 205 kb were treated as one. The total chi-square value was 49.5. In a qui-square test with 5 degrees of freedom, the probability of exceeding 16.7 is 0.005 (0.5%) if the hypothesis is correct.

e) Strain MG1614 had two 175 kb fragments which could not be differentiated on PFGE gels. Accordingly, insertions into these fragments were treated as one class.

Despite the somewhat uneven distribution of TV32 insertions into the *Lactococcus lactis* ssp. *lactis* MG1614 chromosome it was concluded that the Tn*917* derivatives are very useful tools for the genetic analysis of lactic acid bacteria, since it was also found in further experiments that a large number of insertion sites in addition to those mentioned above, could by found with these transposon derivatives.

The above *Lactococcus lactis* ssp. *lactis* MG1614 clone designated 63b was deposited on 21 December 1992 with the DSM-Deutsche Sammlung von Mikroorganismen und Cellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany under the accession number DSM 7361.

EXAMPLE 4

Production of a collection of Tn*917* insertions in Lactococcus lactis

In order to prepare a collection of Tn*917* insertions in *Lactococcus lactis*, the following procedure was followed:

A single colony of a pTV32-containing *Lactococcus lactis* ssp. *lactis* strain MG1614 was inoculated into GM17 medium and grown for 8 to 10 generations with selection for Cm^r. One per cent of these cells were grown for 8 to 10 generations in GM17 medium with selection for Em^r. The temperature was kept at 30°C. The resulting cells were plated onto GM17 agar plates with selection for Em^r. 19 colonies were randomly picked and preparation and digestion of genomic DNA *in situ* in agarose blocks were done as described in Example 3. Figure 5 and table 3 show that 12 out of 18 (digestion one clone resulted in fragments which could be visualized as discrete

bands) clones had the transposon inserted at the same location on the chromosome indicating that the culture was dominated by a single integrant.

Table 3. *L. lactis* ssp. *lactis* MG1614 TV32 integrants from a culture containing a dominant integrant

Group	TV32 target: chromosomal *Sma*I fragment (kb)	Fragment length (kb) of *Sma*I-digested target fragments with inserted TV32[a)]	Group member (integrant No.)
1	600	540 + 70 (= 610)	E15
2	600	475 + x	E4
3	600	400 + 210 (= 610)	E13, E16
4	310	190 + 140 (= 330)	E1, E3, E6, E7, E8, E10, E11, E12, E14, E17, E18, E19
5	200	x + x	E9 [b)]
	140	x + x	
6	175	160 + x	E2

a) indicates a fragment that could not be detected on PFGE gels

b) Double integrant

To circumvent a dominant integrant in a culture, the following procedure was selected:

Strain MG1614 was transformed with pTV32 as described in Example 1. The transformed cells were plated onto SGM17 agar plates containing 1 μg/ml of erythromycin. Following incubation at 30°C for 48 hours, 20 plates each with about 100 colonies were replica-plated onto plates of GM17 agar with selection for Em^r. The replicated plates were incubated at 30°C for 30 hours. The replication step was repeated and the

colonies were washed off and pooled. From the pooled culture, 18 integrants were randomly selected and analyzed by PFGE as defined above. On the basis of the location of the Tn*917* insertions on chromosomal *Sma*I fragments, the 18 integrants were divided into 13 groups of which none contained more than 2 insertions (Figure 6 and table 4). It was therefore concluded that the pooled culture contained a collection of quasi-randomly transposon TV32-insertions in strain MG1614.

Table 4. *Lactococcus lactis* ssp. *lactis* MG1614 TV32 integrants from a culture containing quasi-random TV32 insertions

Group	TV32 target: chromosomal *Sma*I fragment (kb)	Fragment length (kb) of *Sma*I-digested target fragments with inserted TV32[a)]	Group member (integrant No.)
1	600	540 + 70 (= 610)	K10, K20
2	600	530 + 80 (= 610)	K3, K12
3	600	520 + 90 (= 610)	K9, K18
4	600	510 + 100 (= 610)	K13
5	600	470 + 120 (= 590)	K14
6	600	460 + 140 (= 600)	K17
7	600	375 + 220 (= 595)	K4, K6
8	310	185 + 140 (= 325)	K2
9	200	175 + x	K11
10	140	110 + x	K1
11	140	105 + x	K5, K16
12	140	x + x	K7
13	120	x + x	k8

a) indicates a fragment that could not be detected on PFGE gels

Sterile glycerol was added to the pooled culture at a concentration of up till 25% and this mixture stored -80°C.

A pooled culture containing a collection of quasi-random LTV1 insertions in *Lactococcus lactis* ssp. *lactis* MG1363 was prepared essentially as described above. However, before the washing off and pooling of the colonies the following was carried out:

320 µg/ml of X-gal was added to the plates used for the second replication. 242 colonies with varying blue intensities were seen on the second replication plate. In contrast less than 5% of these colonies were blue on GM17 agar plates containing 40 µg/ml of X-gal incubated for more than 48 hours. (40 µg/ml of X-gal is the standard concentration for identification of *lacZ* expression in *E. coli*). Each of the 242 blue colonies appearing on the plate containing 320 µg/ml of X-gal were restreaked to obtain single colonies on GM17 containing 1 µg/ml of erythromycin and 320 µg/ml of X-gal followed by restreaking once on the same medium. A single colony from each of these subcultures was inoculated into liquid GM17 medium supplemented with 1 µg/ml of erythromycin and incubated overnight at 30°C and sterile glycerol added at a concentration of 25% to each of these subcultures for storage at -80°C. These 242 clones are referred to in the following as promoter fusion collection no. 1 (PFC-1).

One of the *Lactococcus lactis* ssp. *lactis* MG1363 PFC-1 clones with the designation P139-170 was deposited with the DSM-Deutsche Sammlung von Mikroorganismen und Cellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany on 21 December, 1992 under the accession number DSM 7360.

EXAMPLE 5

Identification and cloning of regulatable promoters from the *Lactococcus lactis* ssp. *lactis* chromosome

The collection of quasi-random Tn*917* insertions in the *Lactococcus lactis* ssp. *lactis* MG1363 chromosome prepared as described in Example 4 (PFC-1) was used in this experiment which was designed as a screening for the presence of regulatable promoters in these fragments.

Temperature/growth phase regulated *lacZ* expression

Each clone from PFC-1 was streaked onto a duplicate set of GM17 plates containing 1 μg/ml of erythromycin and 320 μg/ml of X-gal. The streak pattern is shown in Figure 7. On one set of plates, the clones were streaked and incubated at 15°C on day 1. On the second set of plates, the clones were streaked and incubated at 30°C on day 4. On day 5, both sets of plates were inspected. Three main types of *lacZ* expression were observed for the PFC-1 clones:

(i) Type 1T showing high *lacZ* expression (dark blue streak) at 30°C and low or no *lacZ* expression (light blue or white streak) at 15°C

(ii) Type 2T showing similar level of *lacZ* expression at the two temperatures

(iii) Type 3T showing low or no *lacZ* expression at 30°C and high *lacZ* expression at 15°C.

Out of a total of 242 clones tested, 23 were of the 1T type, 215 of the 2T type and 4 clones were of the 3T type. Due to the prolonged growth period at 15°C, it is not possible to determine whether the regulated *lacZ* expression is a function of the growth phase/-rate and/or of the temperature.

Arginine/pH-regulated *lacZ* expression

Each clone of PFC-1 was streaked onto a set of M17 plates containing 0.1% of glucose, 0.5% of arginine, 1 μg/ml of erythromycin and 320 μg/ml of X-gal and onto a set of GM17 plates containing 1 μg/ml of erythromycin and 320 μg/ml of X-gal using the same streak pattern as described above. Both sets of plates were incubated at 30°C for about 30 hours. Three main types of *lacZ* expression were observed on the incubated plates:

(i) Type 1A showing high *lacZ* expression on plates without supplementation with arginine and low or no *lacZ* expression on plates supplemented with arginine

(ii) Type 2A showing similar *lacZ* expression irrespective of arginine supplementation

(iii) Type 3A showing low or no *lacZ* expression on plates without arginine and high *lacZ* expression on the plates supplemented with arginine

Out of 242 clones tested, 21 were of type 1A, 219 were the 2A type and 2 clones of the 3A type. The pH of sterile GM17 is about 6.8. The pH in GM17 medium inoculated with *Lactococcus lactis* ssp. *lactis* and incubated overnight is about 5.0. However, the pH in M17 supplemented with 0.1% glucose and 0.5% arginine inoculated with *Lactococcus lactis* ssp. *lactis* and grown overnight exceeds 9.0. Accordingly, the regulated *lacZ* expression observed is a function of arginine concentration and/or pH in the medium.

NaCl/ion strength regulation of *lacZ* expression

Each clone of the PFC-1 collection was streaked onto a set of GM17 plates supplemented with 1 μg/ml of erythromycin, 320 μg/ml of X-gal and 2% of NaCl and on a set of plates with the

same medium but without NaCl using the same streaking pattern as defined above. Both set of plates were incubated at 30°C for about 30 hours. Two main types of *lacZ* expression were observed:

(i) Type 1S showing high *lacZ* expression on plates without NaCl and low or no *lacZ* expression on plates supplemented with NaCl

(ii) Type 2S showing similar *lacZ* expression on both types of plates

Out of 242 clones tested, 87 were of the 1S type and 155 of the 2S type.

When a clone from PFC-1 has been shown to have regulatable *lacZ* expression, an insertion point or a range on the *Lactococcus* chromosome is defined where an inserted gen will be regulatably expressed in *Lactococcus*. For example, the clone designated P139-170 of PFC-1 is of type 3T, type 1A and type 2S which indicates that the *lacZ* gene resides at a position where expression of an inserted gene is suppressed partly or totally at 30°C and on M17 plates supplemented with arginine. However, the gene expression at this position is high at 15°C on GM17 plates. The gene expression level on GM17 plates is unaffected by the tested concentration of NaCl.

Physiological investigation of the regulatable *lacZ* expression of P139-170 clone

P139-170 was shown to be of the type 1A. The following pre-experiment was carried out to study the pH dependence of *lacZ* expression in this clone.:

Six fermenters each containing 1 litre of GM17 medium supplemented with 1 µg/ml of erythromycin were set to operate at 30°C. The fermenters in duplicate were set to operate at pH

5.5, 6.5 and 7.5, respectively using 5 M sulphuric acid or 5 M sodium hydroxide. One of the duplicate fermenters was inoculated with 1% overnight culture of H25A (strain MG1614 containing an LTV1 insertion on the chromosome and capable of expressing β-galactosidase on GM17 agar regardless of added arginine or NaCl) and the other duplicate fermenters were inoculated with 1% of an overnight culture of P139-170.

The growth of the clones in the fermenters were followed by measuring OD_{600} and plating onto GM17 plates +/- 1 μg/ml of erythromycin. The growth curve [$\log(OD_{600})$ versus time] were almost similar for the clones in all of the six fermenters. At an OD_{600} of 2.0, 40 ml of culture from each fermenter was concentrated 10 times and treated twice in a French press. The lysed solutions were subjected to the β-galactosidase activity measurement procedure as described by Miller, 1972, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. Unfortunately, a procedure for storing solutions without loosing β-galactosidase activity failed. Therefore, only results from visual inspections of colour development in the β-galactosidase activity measurement were available:

pH	H25A	p139-170
5.5	+	+
6.5	+	-
7.5	+	-

+: β-galactosidase activity present
-: β-galactosidase activity absent

Based on this experiment it was concluded that *lacZ* expression in P139-170 was a function of pH in the growth medium. However, it cannot be excluded that the content of arginine in the medium might also have a regulatory effect on the promoter function.

From selected integrant PFC-1 clones where regulated β-galactosidase expression was identified, DNA adjacent to the *erm* (or *lacZ*) proximal end was cloned using the following procedure:

Total DNA was extracted from a clone according to the method as defined in Example 2. About 1 μg DNA was digested with 50 units *Eco*RI and incubated for two hours at 37°C. Phenol and chloroform extraction and ligation in 200 μl of ligation buffer containing 50 units of ligase was carried out as described by Maniatis (reference 34). The DNA was precipitated by adding three volumes of ice cold ethanol and 1/10 volume sodium acetate, followed by centrifugation at 10.000 x g for 30 minutes. The DNA was resuspended in 20 μl of TE (1mM EDTA, 10 mM Tris hydrochloride [pH 8.0]). 10 μl ligated DNA solution was used for $CaCl_2$ transformation as described in reference 17, of *E. coli* DH5α (F-, *end*A1, hsdR17(r_k-,m_k+), *sup*E44, *thi*-1, *lacΔU169*, *rec*A1, *gyr*A96, *rel*A1, Φ80 d*lac*ZΔM15). About 1.5 x 10^3 transformants per μg DNA were obtained.

EXAMPLE 6

<u>The construction of a promoter-probe vector for lactic acid bacteria</u>

A useful tool for analysing the conditions that turn on a gene and measuring the level of expression, is a promoter probe. For *Lactococcus*, pGKV210, a promoter-probe vector based on chloramphenicol acetyl transferase and driven by the pWV01 replicon has been constructed (van der Vossen et al., 1985). Unfortunately, this vector only provides slightly enhanced chloramphenicol-resistance when promoters are cloned into it (van der Vossen et al., 1987). Translation of mRNA containing the *cat-86* gene is activated by chloramphenicol (Alexieva et al., 1988) so that the level of enzyme measured is dependent on two factors, the promoter strength and acti-

vation efficiency. In addition, the pWV01 replicon replicates by rolling-circle replication, and is therefore susceptible to size-dependent segregational instability (Kiewiet et al. 1993).

A promoter-probe vector for *Lactococcus* and assumingly other lactic acid bacteria was constructed based on the β-galactosidase genes of *Leuconostoc mesenteroides* subsp. *cremoris*, the *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* citrate plasmid replicon and an erythromycin-resistance marker. This vector is named pAK80. Cloning of the promoter for the tRNA cluster adjacent to the *tma* gene of CHCC285 showed that this vector functions. The resulting construction, pAK90, produces extremely high levels of β-galactosidase in MG1363.

The β-galactosidase genes from *Leuconostoc mesenteroides* subsp. *cremoris* was cloned and found to be nearly identical to the β-galactosidase gene from *Leuconostoc lactis* (David et al., 1992). Both genes have been shown to be expressed in *Escherichia coli* and in *Lactococcus lactis* strain MG1363. The promoter of the β-galactosidase gene was deleted by polymerase chain reaction (PCR) and replaced with a polylinker, allowing cloning of various DNA fragments and testing for promoter activity. This construction was cloned into a shuttle vector containing the *L. lactis* subsp. *lactis* biovar *diacetylactis* citrate plasmid replicon, the pACYC184 replicon for *E. coli* and a selectable marker (erythromycin-resistance) for both organisms. Cloning of a tRNA promoter into the polylinker gave high levels of β-galactosidase in MG1363, proving that the vector works as planned.

A. Materials and methods

1. Bacterial strains, plasmids and media.

MG1363 which is a plasmid-free *Lactococcus lactis* strain (Gasson, 1983). *Escherichia coli* DH5α [*supE44 lacΔU169 hsdR17*

recA1 endA1 gyrA96 thi-1 relA1 Φ80lacZΔM15] (Hanahan, 1983) was used for cloning.

The cloning vectors and relevant markers which were used were: pVA891 [erythromycin resistance; Em^R] (Macrina et al., 1983), and pIC19H [ampicillin resistance; Amp^R] (Marsh et al., 1983). The various plasmids constructed during the construction of the promoter-probe vector are described in the following.

Lactococcus strains were grown at 30°C in GM17 medium. *E. coli* strains were grown in LB medium at 37°C. Antibiotics were used at the following concentrations: for *E. coli*; erythromycin, 250 μg/ml; and ampicillin 50 μg/ml; for *Lactococcus*; erythromycin, 1 μg/ml.

2. Plasmid preparations and transformations.

Plasmid DNA for sequencing and electroporations was prepared with the Qiagen plasmid kit (Diagen, Dusseldorf, Germany).

Small scale plasmid preparations from *Lactococcus* were done essentially according to Israelsen et al. 1993.

Plasmids were introduced into MG1363 by electroporation of glycine-grown competent cells essentially according to Holo and Nes, 1989.

3. β-galactosidase assays

Promoter activity was determined by carrying out β-galactosidase assays on overnight cultures grown in G1.5M17 medium. 1 ml of culture was centrifuged at 10,000 x g for 10 min. The pellet was resuspended in 500 μl Z buffer (Miller, 1972). 100 μl of cell suspension was mixed with 400 μl of Z buffer, 12.5 μl 0.1% SDS and 25 μl $CHCl_3$ on a Vortex mixer for 10 seconds.

After Vortex mixing the suspension was treated as described in Example 7. The results are shown in Table 7.

The assay results are stated as Miller units. One Miller unit = $(1000 \times A_{420})/(\text{time} \times \text{volume} \times A_{600})$ (where time is in minutes and volume is in ml).

B. Construction of pAK66.

Two PCR primers were obtained which allowed amplification of the entire replication region of the citrate plasmid. These had the following sequences:

Primer 1 5' TGAATTCAGAGGTTTGATGACTTTGACC 3'
Primer 4 5' GGAATTCCTAACAAAAGACTATTAACGC 3'

Primer 1 corresponds to nucleotides 610-621 and Primer 4 is complementary to nucleotides 2340-2361 of the citrate plasmid replication region (Jahns et al., 1991). Both contain *Eco*RI sites at their 5' end to facilitate cloning. The 1.7 kb amplification product was cloned as an *Eco*RI fragment into pIC19H to produce pKR41. This *Eco*RI fragment was then moved into the unique *Eco*RI site of pVA891 to produce the shuttle vector pAK66 which replicates in *E. coli* and *L. lactis* MG1363. The construction of pKR41 has been described in a manuscript submitted for publication (Pedersen et al., 1993).

C. Cloning of the *Leuconostoc mesenteroides* subsp. *cremoris* β-galactosidase gene

During the course of cloning and sequencing IS1165 from *Leuconostoc mesenteroides* subsp. *cremoris* strain DB1165 we obtained a clone called pSB1 (Johansen and Kibenich, 1992). This clone contained a 5.8 kb insert in the polylinker of pIC19H. Normally, cloning in pIC19H destroys β-galactosidase activity and colonies with inserts are white on X-gal. pSB1 was strange in that it gave blue colonies on X-gal. DNA sequence analysis revealed that the insert in pSB1 contained the β-galactosidase gene of *Leuconostoc mesenteroides* subsp. *cremoris* and that it was nearly identical to that of *Leuconostoc lactis* (David et al., 1992). Only 3 differences were detected in 830 bp sequenced.

D. Construction of pAK67.7

This construction involved the replacement of the β-galactosidase promoter with a polylinker and insertion of stop codons in all 3 forward reading frames and is illustrated in Figure 8. The promoter was removed by PCR using two primers:

lac-1 ATAGATCTGCAGGATCCCGGGTAACTTTGAAAGGATATTCCTC
lac-2 ATTGAGGGTATACGGTGGGCG

The underlined part of lac-1 is identical to the beginning of the β-galactosidase gene and contains the ribosome binding site. The remaining sequence contains a variety of restriction sites including *Bgl*II. The lac-2 primer anneals to the β-galactosidase gene, 20 bp downstream of the unique *Nco*I site. PCR amplification with these primers will amplify from the ribosome binding site to just beyond the *Nco*I site and produce a 360 bp fragment containing several restriction sites at one end, an *Nco*I site at the other end and no promoter or other regulatory sequences from the β-galactosidase

gene. This 360 bp fragment was purified, digested with *Bgl*II and *Nco*I and cloned into *Bgl*II/*Nco*I digested pSB1. The resulting plasmid was named pAK67 and had the following polylinker preceding the β-galactosidase gene:

```
                H
                i
                n              B       B
                d          X   g       Pa      S
                I          h   l       sm      m
                I          o   I       tH      a
                I          I   I       II      I
AAGCTTTCGCGAGCTCGAGATCTGCAGGATCCCGGGTAACTTTGAAAGGATATTCCTCATG

a       K L S R A R D L Q D P G *
b        S F R E L E I C R I P G N F E R I F L M -
c         A F A S S R S A G S R V T L K G Y S S  -
```

DNA sequence analysis revealed that this polylinker was present and that no alterations had been introduced in the β-galactosidase gene by errors during PCR.

As can be seen above, there are two open reading frames that go across the polylinker into the β-galactosidase gene. Since these could potentially interfere with expression of β-galactosidase from promoters inserted into the polylinker, it was decided to introduce stop codons in all three forward reading frames. This was done by obtaining two oligonucleotides with the following sequence:

Stop-1 GGGTCTAGATTA
Stop-2 TAATCTAGACCC

These oligonucleotides are complementary and will anneal to give a 12 bp piece of double stranded DNA containing an *Xba*I restriction site. This small fragment was cloned into the *Sma*I site of pAK67. These oligonucleotides were designed in such a way that the *Sma*I site would be retained, a new *Xba*I site would be present in plasmids with this tiny insert and stop codons would be introduced into the two open reading

frames. The cloning was done by digesting pAK67 with *Sma*I, phosphatase treating and ligating with a mixture of the two oligonucleotides that had been treated with kinase and allowed to anneal to each other. Transformants were purified and those in which the plasmid had gained an *Xba*I site were further analyzed. DNA sequence analysis revealed that one clone, pAK67.7 had the desired structure:

```
                H
                i
                n          B      B
                d     X    g      Pa    S    X
                I     h    l      sm    m    b
                I     o    I      tH    a    a
                I     I    I      II    I    I
                AAGCTTTCGCGAGCTCGAGATCTGCAGGATCCCGGGTCTAGATTAGGGTAACTTTGAAAGGATATTCCTCATG
             1 ---------+---------+---------+---------+---------+---------+---------+--- 73
                TTCGAAAGCGCTCGAGCTCTAGACGTCCTAGGGCCCAGATCTAATCCCATTGAAACTTTCCTATAAGGAGTAC

          a     K L S R A R D L Q D P G S R L G *
          b      S F R E L E I C R I P G L D *      6-galactosidase  M -->
          c       A F A S S R S A G S R V *
```

E. Construction of pAK80

The final step in the production of the promoter-probe vector was the combining of the manipulated β-galactosidase gene with a replicon and selectable marker for *Lactococcus*. This was accomplished by digesting pAK67.7 with *Hind*III and *Sal*I and ligating into pAK66, also digested with *Hind*III and *Sal*I. Among the plasmids produced, was pAK80 which was the promoter-probe vector exactly as originally designed.

The plasmid pAK80 harboured by *Lactococcus lactis* ssp. *lactis* MG1363 was deposited with the DSM-Deutsche Sammlung von Mikroorganismen und Cellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany on 27 August 1993 under the accession number DSM 8496.

F. Testing of pAK80 using two regulatable tRNA promoters

A DNA fragment from *Lactococcus lactis* subsp *lactis* adjacent to the *tma* gene of CHCC285 has been isolated and found to contain a cluster of tRNA genes preceded by a promoter region (Figs. 11 and 12) comprising two potential promoters (PI, nucleotides 107-134; PII, nucleotides 215-242). The PI and PII promoters, contained on a 501 bp *Hin*dIII-*Sca*I fragment isolated from the clone pLN39 was cloned by inserting it into pAK80 digested with *Hin*dIII and *Sma*I, in front of the promoterless *Leuconostoc mesenteroides* subsp *cremoris* β-galactosidase gene. Following ligation, MG1363 was electroporated and the cells were plated on regeneration medium (Holo and Nes, 1989) containing erythromycin and X-gal. A total of seven blue colonies were obtained. Plasmid analysis revealed that all seven had identical plasmids and that each contained the desired insertion in pAK80. One plasmid was isolated and designated pAK90. β-galactosidase assays revealed that MG-1363/pAK90 produced 5000 Miller units of enzyme, while MG-1363/pAK80 produced 1 Miller units. Thus, the region preceding the tRNA genes contains a very strong promoter.

Searching for sequences with similarity to the sequence of the above promoter region (Fig. 13) revealed a consensus sequence of promoters preceding rRNA operons and tRNA operons from *Lactococcus* species including a previously undescribed conserved sequence (motif), AGTT. This sequence ends 5 bp upstream of the -35 region and is not conserved in tRNA and rRNA promoters of *Escherichia coli* or *Bacillus subtilis*. In all *Lactococcus* species where this AGTT motif was found to precede potential rRNA or tRNA promoters, these promoters had all been isolated from plasmids where the promoters were inserted in front of the *cat-86* gene coding for chloramphenicol acetyltransferase. Since this enzyme is expressed poorly in *Lactococcus lactis* resistance to chloramphenicol can only be obtained in this organism by cloning strong promoters in front of *the cat-86* gene. There-

fore it appears that the motif AGTT is found only in strong promoters of *Lactococcus lactis*.

The above promoters PI and PII both contain conserved sequences assumingly involved in stringent control (Fig. 13) and accordingly, these promoters appear to be regulatable promoters.

A 1.0 kb *Hin*dIII-*Eco*RI fragment from pLN39 was inserted into the plasmid pCI3340 digested with *Hin*dIII and *Eco*RI and the resulting plasmid pLN40 was introduced into *Lactococcus lactis* MG1363. pLN40/MG1363 was deposited with the DSM-Deutsche Sammlung von Mikroorganismen und Cellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany on 22 December 1993 under the accession numbers DSM 8858.

G. Conclusions

This Example describes the construction of a novel promoter-probe vector for *Lactococcus* and assumingly other lactic acid bacteria. This vector has several advantages over previously described vectors. It is based on the *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* citrate plasmid replicon, a theta-replicating plasmid, and so is more stable. The reporter gene chosen is not subject to post-transcriptional control so the enzyme levels can be measured without the presence of any inducers. This is in contrast to plasmids based on the *cat-86* gene where chloramphenicol actually activates the translation of the mRNA (Alexieva et al., 1988). Enzyme assays and plate assays for the reporter gene are simple and standard procedures in most laboratories.

EXAMPLE 7

Measurements of β-galactosidase expression in PFC-1 integrants grown in liquid medium under controlled conditions

Lactoccus lactis ssp. *lactis* MG1363 PFC-1 clones (LTV1 integrants) as defined in Example 4 are usually designated P139- followed by a number indication, e.g. P139-170. In the following, however, PFC-1 integrants are termed only by their number, e.g. 170. In this study was also included the LTV1 integrant in *Lactoccus lactis* ssp. *lactis* MG1614, mentioned in Example 5 under the designation H25A. However, in the following, this integrant has been designated as SB.

The following experiment was carried out with the aims of studying the pH dependence of *lacZ* expression of the two integrants 170 and SB.

Integrant 170 was shown to be of type 1A whilst integrant SB apparently did not belong to this group. Both integrants are of type 2S which means that the expression of β-galactosidase on GM17 plates is not affected by 2% NaCl.

Four fermenters each containing 1 litre of G1.5M17 medium, i.e. 1.5 x M17 broth (Sigma Chemical Co.) containing 0.5% glucose and supplemented with 1 mg/l erythromycin were set to operate at 30°C. Stirring was kept at 150 rpm without active supply of air/O_2. The fermenters in duplicate were set to operate at pH 5.2 and 7.0, respectively using 5 M hydrochloride and 5 M sodium hydroxide. One of the fermenter duplicates was inoculated with 1% of an overnight culture of integrant SB and the other duplicate was inoculated with 1% of an overnight culture of integrant 170.

The fermentations were run for 45 hrs and the growth was followed by measuring OD_{600}. At selected OD_{600} values and time intervals β-galactosidase activity was measured as follows: 10 ml aliquots from each fermenter were centrifuged at 10,000

x g at 4°C for 5 minutes. The pellet was resuspended in 1 ml Z buffer (Miller, 1972) and 0.4 ml of the bacterial suspension and 0.1 ml Z buffer was mixed with 12.5 µl 0.1% SDS and 25 µl $CHCl_3$ by means of a Vortex mixer for 10 seconds. The vortexed suspension was placed in a 30°C water bath for 5 minutes and 100 µl of a solution containing 4 mg/ml of o-nitrophenyl-β-D-galactopyranoside (ONPG) in A-medium (Miller, 1972) was added. The suspension was vortexed for 2 seconds and placed in a 30°C water bath.

The time was noted at ONPG addition and again when the enzymatic reaction was stopped by the addition of 250 µl 1 M Na_2CO_3 followed by Vortex mixing and placing of the suspension on ice. After centrifugation at 10,000 x g at 4°C for ten minutes OD_{420} and OD_{550} of the supernatant were measured. If OD_{550} values exceeded 0.050 the suspension was centrifuged again and OD_{420} and OD_{550} of this supernatant were measured. The β-galactosidase activity was estimated by using the following formula:

$$\beta\text{-galactosidase activity} = \frac{522 \times OD_{420}}{\text{time (min)} \times \text{cell vol (ml)} \times OD_{600}}$$

In Fig. 9 the OD_{600} and β-galactosidase activity versus time are shown for integrant 170 at pH 5.2 and pH 7.0. In Fig. 10 the corresponding data are shown for integrant SB. It is clearly demonstrated by the data in these two figures that the expression of β-galactosidase of integrants 170 and SB are oppositely regulated by pH. Integrant 170 turns off the β-galactosidase expression at pH 7.0. In both integrants, β-galactosidase expression is also influenced by the growth phase. This experiment does not exclude that the concentration of arginine in the medium may also have a regulatory effect on the β-galactosidase expression in the two integrants studied.

EXAMPLE 8

Cloning of DNA fragments containing a lactic acid bacterial promoter and assessment of promoter activity in *Lactococcus lactis*

A. Cloning in *E. coli* of *Eco*RI fragments containing *Lactococcus lactis* DNA and the ColE1 replicon from Tn*917*-LTV1 integrants.

Chromosomal *Eco*RI fragments containing lactococcal DNA, *lacZ*, *cat*, *bla* and the ColE1 replicon, were prepared according to the method described in Example 5 from the Tn917-LTV1 integrants listed in Table 5 below. The fragments were subsequently religated and introduced into E. coli DH5α by transformation as described in Maniatis 1982.

The resulting Tn*917*-LTV1 integrant fragment plasmids were termed p[integrant No], e.g. p86, p143 and pSB. All Tn*917*-LTV1 integrants from which the fragments were isolated are in *Lactococcus lactis* MG1363 except SB which is Tn*917*-LTV1 in *Lactococcus lactis* MG1614.

Table 5. Regulation parameters for β-galactosidase expression in selected integrants. The parameters are deduced from plate assays.

Integrant No.	Parameter
86	arg./pH
143	temp./growth rate
159	temp./growth rate
162	arg./pH
163	arg./pH ; pO_2
170	temp./growth rate; arg./pH
172	temp./growth rate
179	arg./pH; NaCl/ion strength

187	temp./growth rate
188	temp./growth rate
189	NaCl/ion strength
192	temp./growth rate; arg./pH
199	NaCl/ion strength; arg./pH
201	temp./growth rate
202	temp./growth rate
222	arg./pH
224	arg./pH
241	NaCl/ion strength
242	arg./pH
SB	temp./growth rate; arg./pH

B. Subcloning of Tn*917*-LTV1 integrant fragment plasmids into the promoter selection vector pGKV210.

pGKV210 is a promoter selection vector which contains an *erm* gene as a selection marker and a promoterless *cat-86* gene preceded by a polylinker (van der Vossen et al, 1987). The *cat-86* gene is expressed if a DNA fragment carrying a promoter is inserted in the right orientation into the polylinker. The level of chloramphenicol resistance conferred to the host depends on the strength of the promoter.

The integrant fragment plasmids all have a *Cla*I site located in the DNA originating from the *lacZ* part of Tn*917*-LTV1. In order to clone the *Eco*RI-*Cla*I fragments from the plasmids, a *Cla*I site was first introduced into the polylinker of pGKV210 in the following manner: The synthetic DNA linker

5' GATCGCCATCGATGGC 3'
3' CGGTAGCTACCGCTAG 5'

containing a *Cla*I site was cloned into the unique *Bam*HI site of pGKV210 as described by Maniatis, 1982. The obtained plasmid was termed pGKV210(ClaI). 50 ng of pGKV210(ClaI) digested with *Cla*I and *Eco*RI was mixed and ligated with 200

ng of purified *ClaI-EcoRI* fragment as defined above. This was done with *ClaI-EcoRI* fragments from the following integrant fragment plasmids: p143, p162, p163, p170, p172, p224, p237, p242 and pSB.

p162 contains an additional *ClaI* site located in the lactococcal DNA. The fragment from the *EcoRI* site of this plasmid to the additional *ClaI* site was inserted into pGKV210(ClaI) All of the DNA recombination work in this Example was carried out according to Maniatis, 1982.

The resulting pGKV210 derivative constructs were termed pGKV210:[integrant No], e.g. pGKV210:143, pGKV210:162 and pGKV210:SB. The pGKV210 derivatives were introduced into *E. coli* MC1000 (F-, *araD139*(Δara-leu)7679, *galU*, *galK*(Δlac)X74, *rpsL*(Strr), *thi*) according to the method as described in Example 5. The pGKV210 derivatives were extracted as described in Maniatis, 1982 from the transformed host strain. For each extracted pGKV derivative, 1 μg of DNA was introduced into *Lactococcus lactis* MG1363 according to the method as described in Example 1. The resulting transformants (pGKV/MG1363 derivatives) were designated pGKV210:[integrant No]/MG1363, e.g. pGKV210:143/MG1363.

The promoter activity of the above cloned fragments and of previously published pGKV210 derivatives in *Lactococcus lactis* IL1403 (van der Vossen et al., 1987) were determined by plating overnight culture of the pGKV/MG1363 derivatives onto GM17 plates supplemented with 5 mg/l erythromycin and increasing concentrations of chloramphenicol. The concentrations of chloramphenicol were 4, 6, 8, 12, 16, and 20 mg/l, respectively. 50 μl of a 10^4 times diluted culture in a 0.9% NaCl aqueous suspension were plated on plates with 4-8 mg/l of chloramphenicol. 100 μl of a 10^4 times diluted culture in 0.9% NaCl were plated on plates containing 12-20 mg/l of chloramphenicol. The plates were incubated at 30°C for about 80 hrs and the maximum concentration of chloramphenicol still

allowing growth was determined. Results are shown in Table 6 below.

Only two pGKV/MG1363 derivatives were resistant to more than 4 mg/l chloramphenicol. However, difficulties in the interpretation of the results were encountered e.g. due to the appearance of small colonies and this assay seems to be inadequate for promoters of medium or weak strength. The pGKV244/IL1403 and pGKV259/IL1403 produce 0,2 and 5.1 units, respectively, when assayed for chloramphenicol acetyltransferase activity (van der Vossen et al, 1987).

Table 6. Maximum chloramphenicol (Cm) levels allowing growth of strain MG1363 harbouring pGKV210 and pGKV210 derivatives.

Plasmid harboured by MG1363	Concentration of Cm (g/ml)
pGKV210	<4
pGKV244	8
pGKV259	16
pGKV210:143	4
pGKV210:162	4
pGKV210:163	<4
pGKV210:170	<4
pGKV210:172	8
pGKV210:224	<4
pGKV210:237	4
pGKV210:242	<4
pGKV210:SB	12

C. Subcloning of Tn*917*-LTV1 integrant fragment plasmids into the promoter selection vector pAK80.

pAK80 is a promoter selection vector which contains an *erm* gene as a selection marker and a promoterless β-galactosidase gene preceded by a polylinker. The construction of pAK80 is described in Example 6.

The following DNA operations and transformations were carried out according to Maniatis, 1982. The integrant fragment plasmids as described above were first subcloned into the cloning vector pGEM-7Zf(+) (Promega) due to the lack of appropriate restriction sites in pAK80. 50 ng of pGEM-7Zf(+) digested with *Cla*I and *Eco*RI was mixed under ligation conditions with 200 ng of purified *Cla*I-*Eco*RI fragments containing lactococcal DNA from an integrant fragment plasmid. This was done with *Cla*I-*Eco*RI fragments from the following plasmids: p143, p162, p163, p224, p242 and pSB, respectively.

p170 contains a *Sal*I site located in the lactococcal DNA. The fragment from the *Cla*I site to this *Sal*I site was inserted into the cloning vector pBluescript II KS (Strategene) which was digested with *Cla*I and *Sal*I. This construct was termed pBluescript:170. Extracted plasmid DNA from this construction was digested with *Xho*I and *Cla*I and ligated to pGEM-7Zf(+) digested with *Xho*I and *Cla*I. The pGEM-7Zf(+) constructions were termed pGEM:[integrant No], e.g. pGEM:143 and pGEM:170 and collectively designated pGEM derivatives. The pGEM derivatives were introduced into *E. coli* strain DH5α as described in Example 5. The DH5α transformants were termed pGEM/DH5α derivatives.

Plasmid DNA from the pGEM/DH5α derivatives were extracted, digested with *Xho*I and *Bam*HI and ligated to pAK80 digested with *Xho*I and *Bam*HI. The resulting constructions were termed pAK80:[integrant No], e.g. pAK80:143 and pAK80:170 and collectively designated pAK80 derivatives. The pAK80 derivatives were introduced into *E.coli* MC1000 as described in Example 5.

The MC1000 transformants were designated pAK80/MC1000 derivatives. The pAK80 derivatives were extracted from the pAK80/MC1000 derivatives. For each extracted pAK80 derivative 1 μg DNA was introduced into *Lactococcus lactis* MG1363 as described in Example 5. The resulting transformants were termed pAK80:[integrant No]/MG1363, e.g. pAK80:143/MG1363 and pAK80:170/MG1363 and collectively designated pAK80/MG1363 derivatives.

The promoter activity of the cloned fragments were determined by carrying out β-galactosidase assays on overnight cultures of the pAK80/MG1363 derivatives grown in G1.5M17 medium. 1 ml of culture was centrifuged at 10,000 x g for 10 min. The pellet was resuspended in 500 μl Z buffer (Miller, 1972). 100 μl of cell suspension was mixed with 400 μl of Z buffer, 12.5 μl 0.1% SDS and 25 μl $CHCl_3$ on a Vortex mixer for 10 seconds. After Vortex mixing the suspension was treated as described in Example 7. The results are shown in Table 7.

Table 7. β-galactosidase activity of strain MG1363 harbouring pAK80 and pAK80 derivatives.

Plasmid harboured by MG1363	β-galactosidase activity (Miller units)
pAK80	1
pAK80:SB	820
pAK80:143	240
pAK80:162	80
pAK80:163	1
pAK80:170	30
pAK80:224	1
pAK80:242	1

It is clearly demonstrated from the above results that the promoter selection vector pAK80 is capable of discriminating even weak promoters, since pAK80:163/MG1363, pAK80:170/MG1363, pAK80:224/MG1363 and pAK80:242/MG1363 appear to be without promoter activity when assayed for chloramphenicol resistance, but when assayed for β-galactosidase activity it is evident that pAK80:170/MG1363 in contrast to the three other pAK80/MG1363 derivatives, has promoter activity.

The following pAK80/MG1363 derivatives: pAK80:SB/MG1363, pAK80:143/MG1363, pAK80:162/MG1363, pAK80:163/MG1363, pAK80:170/MG1363, respectively were deposited with the DSM-Deutsche Sammlung von Mikroorganismen und Cellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany on 27 August 1993 under the accession numbers DSM 8495, DSM 8497, DSM 8498, DSM 8499 and DSM 8500, respectively.

The *Cla*I-*Eco*RI fragments from p172 and p215, respectively, containing the lactococcal DNA, were cloned into pGEM-7Zf(+). The pGEM-7Zf(+) constructions were termed as described above in this Example.

The pGEM-7Zf(+) constructions were digested with *Bam*HI and *Xho*I and ligated to pAK80, also digested with *Bam*HI and *Xho*I. The details of the cloning experiments were as described above. pGEM:172 was digested with *Xho*I and *Bam*HI. The ligation mixture was introduced into *E. coli* DH5α, and the resulting plasmid, pAK80:172, was introduced into *Lactococcus lactis* MG1363. pAK80:172/MG1363 is blue on GM17 containing X-gal which demonstrates the presence of a promoter on the 4.5 kb *Cla*I-*Eco*RI fragment of p172.

The lactococcal DNA segment of pGEM:215 contains an internal *Bam*HI site.The distal *Bam*HI-*Xho*I fragment of pGEM:215 was ligated to pAK80 digested with *Bam*HI and *Xho*I and the lactococcal *Bam*HI-*Bam*HI fragment was ligated to pAK80 digested with *Bam*HI. Each ligation mixture was introduced into *E. coli*

DH5α. The resulting plasmids were designated pAK80:215A and pAK80:215B, respectively. The correct orientation of the *Bam*HI fragment in pAK80:215B was verified by restriction map analysis. A subsequent introduction of pAK80:215A and pAK80:215B, respectively, into *Lactococcus lactis* revealed that none of the plasmids harboured a promoter. This result suggests that a potential promoter on *Cla*I-*Eco*RI fragments from p215 had been inactivated during cloning of the two subfragments or that the promoter responsible for β-galactosidase expression in Integrant 215 is located upstream of the *Eco*RI site.

Measurements on overnight cultures of *Lactococcus lactis* MG1363 containing the plasmids pAK80:SB, pAK80:143, pAK80:162, pAK80:170 and pAK80:172, respectively, are described in Example 13 below. However, in Example 13 these plasmids are designated pSMA332, pSMA337, pSMA338, pSMA339 and pSMA345, respectively.

EXAMPLE 9

Characterization of a *Lactococcus lactis* promoter regulated by external purine compounds.

The *de novo* synthesis of purine nucleotides from small precursors requires in general 10 enzymatic reactions leading to inosine monophosphate (IMP). IMP is used in synthesis of both AMP and GMP. Purine bases and nucleosides, originating intracellularly or from exogenous sources, are converted to nucleotides via salvage pathways, which have been shown to be distinct among different organisms (for review see: Nygaard 1983). Virtually nothing is known about the purine metabolism in the anaerobic Gram-positive bacterium *Lactococcus lactis* other than described (Nilsson and Lauridsen, 1992).

The media used for growth of lactic acid bacteria may contain purine compounds. Such media repress the synthesis of enzymes

used in the formation of purine nucleotides. When the dairies inoculate the cultures in the purine-free milk, this repression is relieved. This regulation pattern of the synthesis of enzymes used in the purine *de novo* pathway can be used commercially. There may be several genes encoding enzymes that is desirable to have expressed highly in milk, but is unwanted during the manufacturing of dairy starter cultures comprising such genes primarily because of growth inhibiting secondary effects caused by the high expression. Therefore, a purine regulated promoter was searched for in *Lactococcus lactis* and the promoter region, from which the expression of *purD* is initiated was isolated. The *purD* gene encodes an enzyme of the purine *de novo* pathway.

Bacterial strains and growth media

The *Lactococcus lactis* strain MG1363 was grown in M17 medium (Oxoid) or in defined medium, DN-medium. This medium is composed as follows (per litre): 100 ml of a 10% salt buffer with the following composition: $(NH_4)_2SO_4$ 10 g, $Na_2HPO_4,2H_2O$ 33.2 g, KH_2PO_4 15 g, NaCl 5 g, NaAcetate,$3H_2O$ 10 g, ion exchanged water ad 500 ml; 900 ml of basis medium containing 1.0 M $MgCl_2$ 10 ml, 0.5 M $CaCl_2$ 1.0 ml, 0.01 M $FeCl_3$ 1.5 ml, ion exchanged water ad 4500 ml, 15 g of agar per litre; 25 ml of 20% carbon source; 25 ml of casamino acids, 20% (Difco); 10 ml of vitamin solution and 10 ml of a 0.8% aspargine solution.

Glucose was used as carbon source in M17 medium and DN medium. Antibiotics used for *Lactococcus lactis*: Erythromycin, 1 mg/l. Purine compounds as supplements were added, when necessary, per l: Adenine and hypoxanthine, 15 mg; guanosine, 30 mg).

DNA manipulation

Lactococcus lactis plasmid DNA was isolated according to Johansen and Kibenich (1992). *Lactococcus lactis* was transformed by electroporation as recommended by Holo and Nes (1989). The use of the *Lactococcus lactis* promoter-probe plasmid pAK80 is described in Example 6.

Results

A 846 bp DNA fragment (Fig. 14) contains the entire *purD* promoter region as well as an adjacent promoter initiating transcription in the opposite direction. This region was fused to the reporter gene (encoding β-galactosidase) in the promoter probe plasmid pAK80 giving pLN71 (*purD* promoter expression) and pLN72 (promoter expression opposite direction). Transforming pLN71 into *Lactococcus lactis* strain MG1363 gives us the possibility to measure the expression of the reporter gene initiated from the *purD* promoter. The results are shown in Table 8.

The plasmid pLN71 in *Lactococcus lactis* strain MG1363 was deposited on 22 December 1993 with the DSM-Deutsche Sammlung von Mikroorganismen und Cellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany under the accession number DSM 8859.

Table 8. Expression of β-galactosidase in pLN71

Strain	act.[a] in DN-medium	act.[b] in DN-medium + A,Hx,GR[c]
MG1363/pLN71	310	5
MG1363/pLN72	23	6
MG1363/pAK80	<2	<2

[a] Cells were grown exponentially at 30°C in DN-medium containing purines, harvested, washed, and resuspended in purine-free DN-medium, and incubated further 1.5 hour. The β-galactosidase activity expressed from the respective promoter was measured.

[b] Cells were grown exponentially in defined medium containing purines. The β-galactosidase activity expressed from the respective promoter was measured.

[c] A, adenine; Hx, hypoxanthine; GR, guanosine

These results show that the expression of the reporter gene encoding the β-galactosidase is regulated by purine compounds in the media, and that the difference is as large as 60 fold in this experiment.

EXAMPLE 10

Measurement of β-galactosidase gene expression in pSMA344/MG-1363 grown in liquid medium under controlled conditions.

The plasmid pSMA344 consists of the 9.7 kb *Eco*RI-*Cla*I fragment from p170 (see Example 8) inserted into the promoter cloning vector pAK80. In Integrant 170, expression of the inserted β-galactosidase gene has been demonstrated to be regulated by pH and growth phase (Example 7). The following

experiment was performed to investigate if the cloned DNA fragment contains the sequences that are necessary for pH regulated expression of downstream genes.

Lactococcus lactis MG1363 harbouring the plasmid pSMA344 was cultivated in two fermenters each containing 1 litre of medium. The fermenters were set to operate at pH 7.0 and 5.2, respectively, by automatic addition of 5 M sodium hydroxide and 5 M hydrochloric acid. Any other parameter (medium composition, inoculum size, stirring rate, temperature etc.) was as described in Example 7. The fermentations were run for 45 hours and the growth was followed by measuring OD_{600} in culture samples. Sampling and harvesting of culture aliquots as well as measurement of β-galactosidase activity were performed as described in Example 5, except that the culture volume harvested and the cell suspension volume added to the assay were varied according to cell density and expected β-galactosidase activity. Figure 15 shows the OD_{600} and the β-galactosidase activity versus time during the fermentation. It is clear from the results that expression from the promoter harboured on pSMA344 is controlled in the same manner as that observed in Integrant 170. In the culture grown at pH 7.0 the β-galactosidase activity per OD_{600} was less than 1.0 Miller unit throughout the fermentation except in the first sample where some activity will be expected to remain from the preculture. In the culture grown at pH 5.2, β-galactosidase activity per OD_{600} increased during logarithmic growth and continued to increase during the first 14-20 hours of the stationary phase. Both the induced and the repressed levels were 5 to 10 times higher than the values obtained in Integrant 170 under the same culture conditions. This was expected, as the gene carried by the plasmid is present in a higher copy number, and as the two β-galactosidase enzymes encoded by the *lacZ* gene (in *Tn917*-LTV1) and by the *lacL-lacM* genes (in pAK80) may have different specific activities.

EXAMPLE 11

Measurements of β-galactosidase activity in selected PFC-1 integrants grown in liquid medium by a standardized procedure

As described in Example 5, a number of integrants were found to show regulated expression of β-galactosidase when grown on plates under varying growth conditions and medium compositions.

The experiments described below were performed to analyze the regulation of β-galactosidase gene expression in 25 selected integrants grown overnight in liquid culture. The regulation parameters analyzed included pH and/or arginine concentration, sodium chloride concentration, and growth temperature.

Growth media and methods

The media used for liquid cultures are listed in the table below. The basic medium for all experiments was 1.5 x M17 broth (Oxoid, Unipath Ltd., UK) containing 1 mg/l erythromycin.

Table 9. Media used for liquid cultures of integrants

Medium	Composition	Final culture pH
G1.5M17	1.5 x M17 broth containing 0.5 % glucose and 1 mg/l erythromycin	5.5-5.8
ArgG1.5M17	1.5 x M17 broth containing 0.1 % glucose, 0.1 % L-Arginine, and 1 mg/l erythromycin	6.6-6.8

5ArgG1.5M17	1.5 x M17 broth containing 0.1 % glucose, 0.5 % L-Arginine, and 1 mg/l erythromycin	7.7-7.8
G1.5M17-NaCl	G1.5M17 containing 1% NaCl	5.5-5.6
G1.5M17-2NaCl	G1.5M17 containing 2% NaCl	5.4-5.5

All cultures were incubated at 30°C except in the experiment for investigation of temperature effect on β-galactosidase expression, where a set of cultures were incubated at 15°C. In the latter case incubation was prolonged to compensate for the lower growth rate.

To secure uniform starting conditions in all cultures, a 5-10 ml preculture of each integrant in liquid G1.5M17 was inoculated with a single colony from GM17 agar (see Example 15 below) and grown to stationary phase by incubation for 12-18 hours at 30°C. From the precultures 10 µl of each strain was inoculated into 10 ml of each medium,and the cultures were incubated at 30°C for 20 hours or at 15°C for 165 hours. A sample for measurement of OD_{600} was taken from each culture immediately before harvest. The cells were harvested by centrifugation (10 minutes at 10,000 x g, 4°C) and washed once in 1 ml ice-cold 0.15 M NaCl. pH was measured in the medium supernatant. In the case of the duplicate cultures grown at different temperatures where the cultures were harvested on separate days, the cell pellets were frozen at -20°C and thawed later for the β-galactosidase activity assay. The cells were resuspended in 1.0 ml Z-buffer (Miller, 1972), and the cell suspension was subsequently used for assays of β-galactosidase activity as described in Example 7, except that the proportion between cell suspension and Z-buffer used in the assay was adjusted in accordance with the enzyme activity to keep the reaction rate within reasonable limits.

Results of β-galactosidase assays on selected integrants grown in liquid culture

The activity found in the same strain on different days varied to some extent. In ten independent G1.5M17 cultures of Integrant SB the measured activities were between 3.9 and 8.0 with a mean of 6.3 and a standard deviation of 1.4. Five independent cultures of 170 in the same medium gave results between 0.9 and 2.8 with a mean of 1.7 and a standard deviation of 0.7. The observed variation may be caused by some influence on the gene expression or the enzyme stability of undetected differences between medium batches. In each of these cases, however, the difference between activities at low and high pH was obviously significant (Table 10). Activities below 0.1 were not determined accurately by the method used.

Table 10 shows β-galactosidase activities measured in cultures of 17 different integrant strains in media with and without arginine. Most of the integrants showing pH and/or arginine regulated β-galactosidase expression had been identified by plate assays. In Integrants 237, 241 and SB such control of expression had not been clearly observed by inspection of plates. A possible reason is that above a certain activity level it is difficult to distinguish between different activities by the plate assay.

Table 10. Expression of β-galactosidase controlled by arginine and/or medium pH as activity in cells from liquid cultures of selected PFC-1 integrants, grown for 20 hours at 30°C from a 1:1000 inoculum

Integrant No.	G1.5M17 (final pH 5.6-5.8)	ArgG1.5M17 (final pH 6.6-6.8)	5ArgG1.5M17 (final pH 7.7-7.8)
86	0.8	18	
142	1	2-3	2.7
159	1	3	
162	18	50	140
163	8.5	0.3	
168	0.3	0.6	
170	1.7	0.05	0.08
179	4	1	
193	7	18	
203	0.7	0.2	
222	9	5	
224	0.4	0.6	5
229	2.0	≤0.1	
237	7	15	
241	2	5	
242	2	0.01	
SB	6	18	36

A blank space indicates that this particular combination of strain and medium has not been tested.

Ten strains in which β-galactosidase activity during growth on GM17-agar plates varied with temperature were grown to the stationary phase in duplicate cultures in G1.5M17 at 30°C and at 15°C. The activities measured in the cells are shown in Table 11.

Table 11. Dependence on temperature of β-galactosidase activity expressed in selected PFC1-integrants grown in liquid cultures, measured after growth at 30°C for 20 hours and at 15°C for 165 hours, respectively, in G1.5M17 from 1:1000 inocula

INTEGRANT NO.	30°C, 20 hrs.	15°C, 165 hrs.
143	0.8	1.5
159	0.6	1.4
170	1.8	11
172	1.4	0.9
187	1.3	1.0
188	1.3	0.9
192	0.14	1.7
201	1.0	0.8
SB	3.9	8.5

Integrants 170 and 192 exhibited the regulation of β-galactosidase gene expression also found in the plate, both giving higher activity at low temperature. For the Integrants SB, 143 and 159, the effect of temperature on β-galactosidase gene expression was opposite to that expected from the results of plate assays, and for Integrant 172, 187, 188, and 201 the effect was weaker than anticipated. It must be taken into account that the cells from the liquid cultures were harvested in stationary phase, whereas the β-galactosidase activity detected in the plate assay is accumulated from both the growth phase and the stationary phase.

Plate assays of PFC-1 integrants had revealed either decreasing β-galactosidase gene expression or no change in response to addition of NaCl to the growth medium. The results of activity measurement in cultures grown in liquid medium containing 1% or 2% NaCl are shown in Table 12. For the integrants 179, 199, 230, and 241 it was expected from plate

assays that NaCl would reduce β-galactosidase activity. Several integrants that had not shown any influence of NaCl on β-galactosidase activity in plate assays were included in these experiment, and results from three of these, namely 224, 229 and SB, are also presented in the Table.

In all strains tested activities had decreased by a factor of 3-30 in media containing extra NaCl, and apparently the strongest effect was on activity in SB. As mentioned earlier, the final pH of the cultures in NaCl-containing media was slightly lower than in cultures grown without additional NaCl. However, this pH difference may not be large enough to account for the clear effect on SB gene expression, nor is it likely to explain the similarity of the effect on all strains tested. More controlled experiments are needed to elucidate the apparent contradiction between the results of the plate assay and the activity measured in liquid overnight cultures.

Table 12. Effect of NaCl in medium on β-galactosidase activity in cultures of selected PFC-1 integrants, grown for 20 hours at 30°C from a 1:1000 inoculum. A blank space indicates that this particular combination of strain and medium has not been tested.

Integrant No.	G1.5M17 (final pH 5.6-5.7)	G1.5M17-NaCl (final pH 5.5)	G1.5M17-2 NaCl (final pH 5.4)
179	3 2	 0.7	0.4 0.09
199	0.12	0.04	0.02
230	0.15 0.3	 0.1	0.01 0.01
241	2		0.3
224	0.5		0.04
229	2		0.14
SB	6	0.6	0.3

The following integrants (host organism: *Lactococcus lactis* MG1363): SB, P139-86, P139-142, P139-143, P139-159, P139-162, P139-163, P139-168, P139-172, P139-179, P139-187, P139-188, P139-192, P139-193, P139-199, P139-201, P139-203, P139-222, P139-224, P139-229, P139-230, P139-237, P139-241, and P139-242 were deposited with the DSM-Deutsche Sammlung von Mikroorganismen und Cellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany on 22 December 1993 under the accession numbers DSM 8834, DSM 8835, DSM 8836, DSM 8837, DSM 8838, DSM 8839, DSM 8840, DSM 8841, DSM 8842, DSM 8843, DSM 8844, DSM 8845, DSM 8846, DSM 8847, DSM 8848, DSM 8849, DSM 8850, DSM 8851, DSM 8852, DSM 8853, DSM 8854, DSM 8855, DSM 8856 and DSM 8857, respectively.

EXAMPLE 12

Sequencing of *Lactococcus lactis* chromosomal DNA upstream and downstream of Tn*917* insertion in selected Tn*917*-LTV1 promoter fusion integrants.

The chromosomal sequence of about 200 bp to 1500 bp upstream of Tn*917* insertion was determined in six selected Tn*917*-LTV1 *Lactococcus lactis* promoter fusion integrants. In one of the selected integrants, the sequence downstream of the transposon insertion was also determined. The sequencing was done to present examples of sites and regions on the chromosome of *Lactococcus lactis* showing regulated expression of inserted promoterless gene(s). Sequencing was performed on both strands essentially as described in the manual for Sequenase Version 2.0 DNA Sequencing Kit from USB, Cleveland, Ohio, USA, using the integrant fragment plasmids (see Example 8) pSB, p170, p143, p242, p224, and p163, as templates and primers as described below. We defined *Lactococcus* DNA located next to the *lacZ* proximal end of Tn*917*-LTV1 to be upstream of transposon insertion. Regardless of which strand being mentioned, to move away from the *lacZ* end is to move

upstream on the *Lactococcus* DNA. The strategy for sequencing upstream on each template was as follows:

1. The first sequence reaction was performed using the primer pp1 (5' GTTAAATGTACAAAATAACAGCG'3) (DNA Technology, Århus, Denmark). pp1 is homologous to a sequence in the *lacZ* proximal end of Tn*917*-LTV1. If the first bp upstream of Tn*917*-LTV1 is designated No. 1, the complementary sequence to pp1 is located at bp No. -58 to No. -80. The obtained sequence, designated pp1-sequence, consisted of about 20 bp of the *lacZ* proximal end of Tn*917*-LTV1 followed by 200 to 300 bp of adjacent, upstream *Lactococcus lactis* DNA sequence.

2. Based on the pp1-sequence the primer p1 was synthesized (DNA Technology). p1 is homologous to a 20 to 24 bp sequence located about 60 bp from the 3' end of the pp1-sequence. The second sequence reaction was performed using the primer p1. The obtained sequence, designated p1-sequence, was an overlap of about 20 bp of the 3' end of the pp1-sequence and extended 200 to 300 bp further upstream on the *Lactococcus lactis* DNA.

3. Based on the p1-sequence two primers, p2 and p1r, were synthesized (DNA Technology). p2 is homologous to a 20 to 24 bp sequence located about 60 bp from the 3' end of the p1-sequence and p1r is homologous to the complementary *Lactococcus lactis* DNA sequence located about 250 bp upstream of transposon insertion. The third and fourth sequence reaction was performed using the primers p2 and p1r, respectively. Using the primer p2 the obtained sequence, designated p2-sequence, was an overlap of about 20 bp of the 3' end of the p1-sequence and 200 to 300 bp further upstream on the *Lactococcus lactis* DNA. Using the primer p1r the obtained sequence, designated p1r-sequence, was complementary to the pp1-sequence.

4. Additional sequence reactions were performed using the primers p3, p4, etc., each primer homologous to a sequence located about 300 bp upstream of the previously used primer.

Also, sequence reactions were performed using the primers p2r, p3r, etc., each of which are homologous to a sequence located about 300 bp upstream of the previously used primer.

5. Cloning of the sequence located downstream from the Tn*917*-LTV1 insertion in Integrant SB: When the Tn*917* derivative, Tn*917*-LTV1 is used for transposon mutagenesis, the DNA located upstream of the insertion point can easily be cloned in *E. coli* as described in Example 5. However, this cloning method can not be used for cloning DNA located downstream of the Tn*917*-LTV1 insertion. However, using the Inverse Polymerase Chain Reaction strategy (Ochman et al. 1988) the DNA located downstream of the transposon in Integrant SB was amplified and cloned in *E. coli* in the following manner:

60 ng of chromosomal *Lactococcus lactis* MG1614 DNA was completely digested with *Eco*RI. The digested DNA was phenol/chloroform extracted and precipitated with NaAc and EtOH. The DNA was subsequently ligated in a total volume of 20 µl. This diluted concentration favours the formation of monomeric circles. From this ligation mixture a 5 µl sample was taken and a PCR amplification was performed in a total volume of 100 µl. The two primers
BA24:(5'CCAGTCAACTTTAAAACATAACC3') and
BA21:(5'CTCACTGGTCACCTTTATCC 3') were used for the PCR amplification. A GeneAmp DNA Amplification Reagent Kit from Perkin Elmer Cetus, 761 Main Ave., Norwalk, CT 06859 was used. The concentration of reaction buffer, dNTPs and Taq polymerase was as described in the protocol from the manufacturer. The final concentration of the primers in the reaction mixture was 10 ng/µl. The following temperature profile was used: Denaturation at 94°C, 1 min.; annealing at 53°C, 1 min.; extension at 72°C, 2 min. The total number of PCR cycles were 40.

When 10 µl PCR reaction product was analyzed on an agarose gel, one specific band of about 1400 bp was observed, indi-

cating the cloning of about 1100 bp downstream of the Tn*917* insertion in Integrant SB.

The 1400 bp fragment from the PCR reaction was ligated to the pT7Blue(R) vector (Novagen, Madison, Wisconsin, USA) under standard ligation conditions as described by Maniatis et al. 1982. The ligation mixture was introduced into *E. coli* DH5α The resulting plasmid was designated pSBC1.

The subsequent sequence reactions were performed using the same strategy mentioned above in paragraphs 2, 3 and 4.

In the following, DNA sequences upstream of Tn*917*-LTV1 insertion in Integrants SB, 170, 143, 242, 224, and 163, respectively, are shown [(i) - (vi)]. For Integrant SB a DNA sequence downstream of Tn*917*-LTV1 insertion in Integrant SB is also given. The site of transposon insertion and orientation of Tn*917*-LTV1 is shown by [lacZ--Tn917-LTV1-] inserted into the sequences.

(i) The DNA sequence of 117 nucleotides upstream of the *lacZ* proximal end of Tn*917*-LTV1 and a DNA sequence of 1.083 nucleotides downstream from the *lacZ* distal end of Tn*917*-LTV1 in Integrant SB.

A putative transcription terminator is indicated with lower case letters and the -35 and -10 consensus sequences of the promoter, PSB is underlined.

```
1(5')CTGGTCACCT TTATCCATTG AAAATTGATA ACAAAGGATT ACAAGTagaa
                                                  -35
51   gaatctgtat tttaatacag gttctttttG TTGATTATTT TATAGATAAA
            -10
101  ATGATATAAT CATTAAA [lacZ---------Tn917-LTV1----------
     ----------------]GCA AAAAAGAATG TAAAGTAGTT CACTAACTTT
151  CGTTTTATTT GTCAGAATAA GGTTTTTGAT TTATCATTTT TTTAAAGTTA
201  AAAGTAATGA ATTATTAAAT TTCTTCTAAT GACAAAAAAT GTGATTTAAA
251  TGAGAAACCA CGATTGCCCT ACTGTCCGCT TTTTTAAAGC AAGAGTTTAT
```

301 AAAGAAAAGG AAACTCAAAT GACTCAAACA AAAAAGGCAA AAGTCAGAAA
351 TCTGATTATT GCTGCGATGC TTACTGCACT TGGAATTTTA ATTCCAATGA
401 TGATGCCGGT TAAACTCATT ATTGGCCCAG CCTCATTCAC GCTTGCTGCA
451 CATGTTCCGG TAATGGCTGC CATGTTTTTC AGTCCACTTA TGACTGCTTT
501 TGTTGCTCTG GGAACAACTC TCGGATTCAT GATTAGTATT CCGGTGCCAA
551 CAATTTGGTT GCGCGCGCTG ATGCACCTTC CTGTAATGAC TGTTGGTGCC
601 TATGTCTTGA AAAAATATCC AGAATTTGTT CATCAAAAAG TTAAAATCCA
651 AATCTTTAAT TTTATTCTCG GTATTTTTCA TGCTGGTTTG GAAACTTTAG
701 TTGTTTATGC TTTTTTATTCT CTAGGATTTG CGAATATTGA GCAAGGTGCT
751 TTATTGAACT TCCTCTTATT GATTGCTCTT GGAGGACTTG TCCATAGCAT
801 GATTGACTTC AACTTAGCGC TTGGTTTGGG TAATGTTTTG AGTAAAGCCT
851 TTCCTATTGA CATCTTTGAT AAAGCTAAAA ATCTTGTGAA TAAAAAGAAA
901 GTTAAAGCCG AAATTTAAGA CAAAATTGTC ATCTTTAATA GAAAATGATA
951 AAATAAGGTT ATGATAAAAG AAACTGATCT TGAAAATATC CCAGATTTAC
1001 TGATTAAATT TAATGAACCC CTATCAAATT ATACTTACAC AAAAGTAGGA
1051 GGACCAGCTG ATATTCTGGC TTTTCCGGCT ACAATAGAAG CATTGACAGA
1101 ACTGTCAGCA AAAGCGAACA GACTGATACA CCGGTTACAG TTCTTGGAAA
1151 TGCCTCAAAT TTGATTGTTC GTGATGGTGG AATTC(3')

(ii) The DNA sequence of 1.430 nucleotides upstream of the *lacZ* proximal end of Tn*917*-LTV1 in Integrant 143.

1(5')CATCATTTAT TTCAAAGTAT AAAAAAAATC AATGGAAAAG TTGTATGGAT
51 TAATATCAGT TTTCTTTTCG TATTATCACT AATTCCTATT TTTTCAAACT
101 GGGTATCAAT ATATCCCAAT TCATTTATTC CAGAACTAGG TTATGTCATT
151 ATCTTTTTCT TTGGAAACTT CATCTACTTT CTATTAACAA GGGAATTATT
200 AAAAATTAAT GGTCACCGTA AAACTTCTGA ATCAACTGTA AGAAAAAATA
251 TCATCAGTGT TGGACTTAAT GTCATTAGCA TTATTCTTGG ATATTTTATT
301 GCACCGGTGA TTATGCTCAT TGCTTCGGCG TTGATTTTTT CAATGTGGGT
351 CATTCCAGAT AAGAACATTG AAAAAATGTT TAAATAAGTA TTTTATAAAA
401 ATAGAATTTG TATCAAGAAA AATTTGGAAA AACTGACTAA ATTGTCTGTC
451 AGTAAATTAA ATATAAATTG AGGAGAAAAT AATGATTAAA GCATACATTA
501 AATATTGGAA AAAAGCAGGC GATTTCAAAA CATATTCAAG TCGTTCAGAT
551 TACTGGTGGG TTTTCTTGGC GAATTTCATT ATCTTTGCTA TTCTAAGCTT
601 TTTTAATTTT ATGATTATGA TACCAAGAGC TGCCAAAATC ATGAATCAAG
651 CAGGTGACTC ATCTCAAACA GAAATCATTC GACAAGTCAC GGATTTATAC
701 ACAAATCCTA CAGGTGGAGC ATTAGTGATT ATTATCATTA CAGCTATTGC
751 TGGTTTGGCT ATTCTTATTC CAAGCGTTAG TCTGACAGCC CGTCGTTTGC

801 GAGATGCACG TCTTCCTTGG TGGATTTCTC TTATCTTTGG TTTAGCAGCC
851 ATTTATGGTT TACTTACAAT GTTTATTCAT CAAGAAATGC TTCAACAGTT
901 AGGATTCATT TTTAACTTAA TCACTTTCAT TGTCTATATC CTCTGTCTTT
951 TCCCAACAAA ATATGGAGTT GAGGAAGAAG ATGACTCAAG ATCTTATGAA
1001 TAGTACAAAA AAGAAAGGTA AAATATGATA CAAGCTTATA AAAAATATTG
1051 GCAAGGGACT TTTGTTTTCA ATAAAAGAAC AAGTCGTAAG GATTTTTGGA
1101 TGGCTTTATT CACCCATCTG ATTATTTTTG TGGTTTTACT AAAGGGCTAT
1151 AATTTTTTTA ACGGATTGGG TTATTTCCCA CTGTCAGTTT TATGGCAATC
1201 AATCGGTTCA TTTTTACTTT GGCTTTTGTG GATATATTTT TTAGGAAGTT
1251 TACTAGCCTT CTTGGCCATA ACAGTTCGAC GATTAAATGA TACTGATTTG
1301 CCTTGGGGAT TAGTATTTCT AAATCTTGTT TTTGGCTTAG GAACTCTTGT
1351 ACTATTGGTT CTCAATTTAT TTCCAAGTTC TCCTAAAAGA GACAAGTTTA
1401 AAGAGTTTGA ATTAAAAAAT AGTTCTAATT [lacZ-Tn917-LTV1](3')

(iii) The DNA sequence of 994 nucleotides upstream of the *lacZ* proximal end of Tn*917*-LTV1 in Integrant 163.

1(5')TTTTCATTGC CTACATTGGG ATTAAAAACG CTGGAATTTT GCGCTTCATC
51 GCTGACCCAG GAACTTATGT GAACAATCAC GGAACAATTA CAGCAAATTC
101 ATCAATTGTT CCAGAGCTTG TAACTTTTAA TAACCCAGGA GTGTTGGTAG
151 CACTTGTTGG GATTGTCGTG ACAATGTTCT TTGTCATTCG TAAATGGCGG
201 GCAGGGATTT TGCTTTCAAT CTTGGTAACA ACTATCTTGG CTCTTTTGAC
251 TGGCGTGGTT AAAGTTGATG TGAATACTTT ATTTGCTGAA AATAATTTGG
301 GGACTGCAAT CAATCAAATG GGAACAACCT TTGGTGCAGC ATTTGGTCCA
351 AAAGGATTTG GTTCTTTATT CTCTGATTCA TCACGTTATA TTGAAGTATT
401 AATGACAGTT CTTGCTTTCT CATTGACTTC AATCTTTGAC CCAATCGGAA
451 CTTTCATCGG AACTGGTCGC GCGACAGGAA TCTTTACTGA TGAAGATTTG
501 AAAGACATGG AAACAAGCCA TGGTTTCTCA TCAAAAATGG ACAAAGCTTT
551 GTTTGCTGAC ATGATTGCTA CTCCAATCGG AGCAATTTTC GGAACATCAA
601 ATACAACCGT TTATGTTGAG TCTGCTGCCG GAATCGGTGC AGGAGGACGT
651 ACTGGTCTTG CATCAGTTGT AACAGCAATT ATGTTTGCTA TCTCAAGCTT
701 GTTCTTACCA CTTCTTGCGA TTGTTCCAAC ACAAGCAACA GCACCAATTT
751 TGATTATCGT TGGGATGATG ATGCTTGGTT CATTTAAAGA AATTAAATGG
801 GGTGATTTGA CAGAAGCGAT TCCTGCTTTC TTCGCCTCAG TATTCATGGG
851 ACTTGCTTAT TCAATCTCTT ACGGGATTGC AGCTGGATTT ATCACTTATA
901 TCCTTGTCAA ATTATTCACC GGAAAAGTGA AAGAAATTAA ACCTGTAATT
951 TGGGTCGTTG CTCTCTTGTT CTTAATTAAC TTTGGGGTCC CGAG [lacZ-

-Tn917-LTV1-](3')

(iv) The DNA sequence of 1.120 nucleotides upstream of the *lacZ* proximal end of Tn*917*-LTV1 in Integrant 170.

```
    1 (5')TGTCGTTTTT TCTTCCAAAT AAACGACAAT ATGATTGTAC TGCGCTCGAT
    51    TAGGAAAGAC AAATGGAAAA AGAATCCAGC AAAAATGGAA TAAGCACTCC
   101    AAACCAACTC AGAATAGCCA CCAATGTTTG AAATATTTTA CTCCCATAAT
   151    TCCCTTTTTC AAAATACGGG TCATAAACTA AAGATTTTTT CGCCTCTTCA
   200    CGGCTCAAGT TTTGTTTCAT TTCCGACCTT TCTGAACTTT TCAACCTTTT
   251    ATAGTTATAG TCAATACAAT ACATTTTCTT TAATTATCTC ATTTTTTGTT
   301    CACAAAAGCC ATTTTATGAG TCTATTTTTA ATTACAAAAA ACAGTCAGAC
   351    ACTCTATCAA ACTGCTTTAT ATTTATTATT TATAATGATA ACAGTCGATT
   401    CTCCTTTTTT ATCAACTTTT GCTTTATGCT ATAATTTACA GATAAGAACG
   451    ATCTACCTAA AAAGGTTAAA GGAGTATTAT GATAAAAATT TTAAAAATGA
   501    CTCAAGATGG CTTTGACCAT TATATGTTGT CCGCTATTAA AAATTATGCT
   551    AATGAGAAAG TAAATAATGG AACATGGGAG TCTAAAGATG CCCTTTCAAA
   601    TTCAAAGAAA CAGTATGCAC TCCTGCTTCC CGACGGCTTC AAACTGCTAA
   651    TCATTATTTT TACTCAATTT TTAATAAAGA AGAAAAAATC GGATATATCT
   751    GAAATTTATG AAGAATTTCA AAATCTAGGA TTTGGCTCAA AAACCCTTGA
   801    TTTAGTTGCC GATAAAGCAA AAGAACTTGG ATTCTCTTTT TTGGGACTCC
   851    ACGTTTTTGG AAGTAATTCT AGAGCTTTGC ATGTCTATAA AAAAATGGGA
   901    TTCCAAATTA CCGATATCAA TATGCGAAAA GAACTATGAA TATCCACTCC
   951    ATTTTTGGTT GCCATTTGTT AACGCTGCCT CCTCTCCCTA GTGCTATAAT
  1001    AAAAATGGCC AAAAAAAAAC CATTTTATTG ACTATATTTG CAATTTATTT
  1051    ACACATTATC TTTTCAGAAC CAAAATCTGG CCCATTTTGG AACAGACTTC
  1101    TACTATTTTG TTGTCTAGTA [lacZ-Tn917-LTV1-](3')
```

(v) The DNA sequence of 480 nucleotides upstream of the *lacZ* proximal end of Tn*917*-LTV1 in Integrant 224.

```
1(5')GAATTCTTGA TTCAATGAGA GCTATTATGC TTATCGTCGA ATTAGAAGGT
  51   GCATTTGATA TTAGTCTTCC ACCATCAGAA ATGGACCGTG AAGATTGGAA
 101   TACAGCAAAT AAAATAGCAG CACGCGTTCA GGAAAAAACG GATGAAAATT
 151   AAAATTTTTA GAGCAATTGG CCCACTAATT GCAGCTTTAG TTCTCGTTGC
 200   TTTATTAATA TTTCTCCCTT TTTAACGTTG GAATGAAATA TTCTAAAGAC
 251   CAACTCGTTA AGTTTGCACA GTCACCCTTA AATACACCTA CTTTTACAGG
 301   ATATTCAATT AAGAAACAAG CCTATTCAGA TCCTGAATTT TTACCAGTTC
```

351 TCGGTTCGTC AGAAATGGAA CACGTTGATT CATTTCACCC AAGTGCTTAT
401 TTCAGCAAAT ATAATTCAGG TTTCATACCA TTTTTAGTAG GACAACCCGG
451 AACAACGACA TTAACTCACT TTTTCTATAT [lacZ-Tn917-LTV1-](3')

(vi) The DNA sequence of 853 nucleotides upstream of the *lacZ* proximal end of Tn*917*-LTV1 in Integrant 242

1(5')TTAGAACGTC AATGAGATAG AAAAACAAAA TATTTAAGAA TAAAATGATA
51 CTGTTTTCCT TAACTTAATG ACATTGGGGT ATACCCTGTT GTCCATCAAA
101 AAAAATCTTC TAAAATTATT TTACTCAAAT TGATAGATTA TTTTTATGAA
151 ATGTGTTAAC ATTTATTACT ATCTAAATAG CCAGAAAATT CTACAATAGA
200 GTTATAAATT AATGGAGACT CTATATGAGA AAAAATAAAA CCAAGTTTAT
251 TGCTTTTGCA CTTGCTTAAG CAGTTATTGC AGTAGGTTAC TCAACTGCAG
301 CTTCTGCTGA TTCTGTTACT TCCTCAGATA AAGATACAGT CTCAAATCCA
351 ATTCTGACAA TTACACCTCG TATGAATGTT GAGTTTCAAG GTGGTGGATA
401 TTGGACAAAT ACTTCGCACC TGACCTACAT TCAAAATACA GGTTCTGGAG
451 TACTGTATTA TGACCGAGTA AATCATAAAT ATGTATTTTC ACAAACAAGA
501 GGTGCAATGG GTGCAGCTAT TTATGTTTTT AACGCTCAGG GTGTAAACTG
551 GTATAGAGGA GTACTTTATG TTTAAGAGTA AAAAAAATGA TGAGAAGAAG
601 GTTGAAATAC TCAATTCTAT TGATAAACTT CTTCATCAAG ATGTTGAATT
651 AACAATAGAC GAAAAAGAAA TACTGTTAAA ATATAAAGAG CGGATTCAAA
701 ATTCAAAAAA TATTGAATTT GAACTGATTC ATCTTAGAAA TGCTCTTCTT
751 CCATTTGTTA TAAGTTCGAA ACTTTCCGAA CCTACATTAA ATTTCTATAA
801 AAAAATACGA GCAGATAGAA AAATTAGATG GGGAGAAGGT AGCTCTCTAA
851 TTA [lacZ-Tn917-LTV1-](3')

EXAMPLE 13

Mapping af the promoter, P170 on the 9.7 kb *EcoRI-ClaI* DNA fragment from p170

The following experiments were carried out to map the location of the pH/growth phase regulated promoter, P170 on the 9.7 kb *ClaI-EcoRI* fragment of p170.

The 9.7 kb *ClaI-EcoRI* fragment of p170 was cleaved into subfragments and a restriction map was created (see Figure

16). Appropriate subfragments were subsequently cloned into the promoter probe vector pAK80. However, it was necessary first to create compatible restriction sites on the subfragments and pAK80.

(i) Construction of pSMA344

Cloning of the large 9.7 kb *Cla*I-*Eco*RI fragment from p170 into pGEM-7Zf(+) was done by digesting p170 with *Cla*I and *Eco*RI followed by ligation of the 9.7 kb fragment to pGEM-7Zf(+) digested with *Cla*I and *Eco*RI. The ligation mixture was introduced into *E.coli* DH5α and the resulting plasmid was termed pSMA212. pSMA212 was digested with *Xho*I and *Bam*HI and ligated to pAK80 also digested with *Xho*I and *Bam*HI. The ligation mixture was introduced into *E.coli* DH5α. The resulting plasmid, pSMA344, was subsequently introduced into *Lactococcus lactis* MG1363.

(ii) Construction and cloning of deletion derivatives of the 9.7 kb *Cla*I-*Eco*RI fragment from p170.

Plasmid pSMA342 was constructed in the following manner: pSMA212 was digested with *Cla*I and *Nde*I, the sticky ends were filled in by use of Klenow polymerase as described by Maniatis et al. 1982. The large 8.7 kb fragment [3kb from pGEM-7Zf(+) and 5.7 kb from the *Lactococcus* chromosome] was purified, religated, and introduced into *E.coli* DH5α. The resulting plasmid, pSMA213, was digested with *Xho*I and *Bam*HI and the purified 5.7 kb fragment was ligated to pAK80 also digested with *Xho*I and *Bam*HI. The ligation mixture was introduced into E.coli DH5α and the resulting plasmid, pSMA342, was subsequently introduced into *Lactococcus lactis* MG1363.

The plasmid pSMA343 was constructed in the following manner: pSMA212 was digested with *Cla*I and *Sal*I, the sticky ends were filled in by Klenow polymerase. The 6.2 kb fragment [3kb from pGEM-7Zf(+) and 3.2 kb from the *Lactococcus* chromosome] was purified, religated and introduced into *E.coli* DH5α. The

resulting plasmid, pSMA214, was digested with *Xho*I and *Bam*HI and the 3.2 kb lactococcal fragment was ligated to pAK80 digested with *Xho*I and *Bam*HI. The resulting plasmid, pSMA343, was introduced into *E.coli* DH5α and subsequently into *Lactococcus lactis* MG1363.

The plasmid pAK80:170 (DSM 8500) as described in Example 8 is in the following designated pSMA339.

Plasmid pSMA340 was constructed in the following manner: The cloning of the 6.5 kb *Cla*I-*Sal*I lactococcal fragment from p170 into the cloning vector pBluescript II KS is described in Example 8. This construct being termed pBluescript:170 in Example 8 is designated pSMA201 in the following. pSMA201 was digested with *Nde*I and *Sal*I and treated with Klenow polymerase to fill in the sticky ends. The large 7 kb fragment [3 kb from pGEM-7Zf(+) and 4 kb from the lactococcus chromosome] was purified, religated and introduced into *E.coli* DH5α. The resulting plasmid was termed pSMA202.

pSMA202 was digested with *Xho*I and *Bam*HI, and the 4 kb lactococcal fragment was purified and ligated to pAK80, also digested with *Xho*I and *Bam*HI. The ligation mixture was introduced into *E.coli* DH5α and the resulting plasmid, pSMA340, was subsequently introduced into *Lactococcus lactis* MG1363.

pSMA341 was constructed in the following manner: pSMA202 was digested with *Nde*I and *Eco*RI and treated with Klenow polymerase to fill in the sticky ends. The large 5.5 kb fragment [3 kb from pGEM-7Zf(+) and 2.5 kb from the lactococcus chromosome] was purified, religated and introduced into *E.coli* DH5α. The resulting plasmid, pSMA208 was digested with *Xho*I and *Bam*HI and the 2.5 kb lactococcal fragment was ligated to pAK80, also digested with *Xho*I and *Bam*HI. The resulting plasmid, pSMA341, was introduced into *E.coli* DH5α and subsequently into *Lactococcus lactis* MG1363.

(iii) Assessment in *Lactococcus lactis* of promoter activity on the subfragments of the 9.7 kb fragment from p170

A plate assay for determination of promoter activity of the cloned lactococcal fragments was performed by plating overnight cultures of *Lactococcus lactis* containing the plasmids pSMA339, pSMA340, pSMA341, pSMA342, pSMA343 and pSMA344, respectively, on GM17 supplemented with 1μg/ml Em and 160 μg/ml X-gal. Surprisingly, all cultures appeared blue on these plates, showing the existence of at least one functional promoter on all plasmids. From these results it is evident that at least three promoters are located within the lactococcal 9.7 kb fragment from p170.

The *Lactococcus lactis* MG1363 strains containing pSMA339, pSMA340, pSMA341, pSMA342, pSMA343 and pSMA344, respectively, were streaked on GM17 plates and on ArgM17 plates, respectively. Both type of plates contained 1μg/ml Em and 160 μg/ml X-gal. The platings were done to identify the pH regulated promoter(s) among the three promoters. Based on these assays the β-galactosidase expression arising from pSMA339, pSMA340 and pSMA344, respectively, was found to be regulated by pH/arginine. The β-galactosidase expression arising from pSMA342 was weakly regulated by pH/arginine, whereas the expression from pSMA341 and pSMA343 were unaffected by these factors.

The results demonstrate that the promoter located on the 4 kb *Cla*I-*Nde*I fragment proximal to the β-galactosidase reporter gene, is pH regulated. This promoter is in the following referred to as P170. The plasmid pSMA342, which contains the 5.7 kb lactococcal fragment extending from the *Nde*I site to the *Eco*RI site, most likely contains two promoters, of which the one located proximally to the reporter gene also appears to be pH regulated. However, this regulation seems to be dependent on the 3.2kb *Eco*RI-*Sal*I fragment located upstream. This conclusion is based on the observation that the promoter

harboured on pSMA341 which lacks the 3.2 kb *EcoRI-SalI* fragment, is not regulated by pH/arginine.

Measurements of β-galactosidase expression in overnight cultures of strain MG1363 containing pSMA339, pSMA340, pSMA341, pSMA342, pSMA343, and pSMA344, respectively, were performed as described in Example 7. All cultures were grown in GM17 medium and ArgM17 medium, respectively. Both media were supplemented with 1μ g/ml Em. As a control of regulated β-galactosidase expression, Integrant 170 was included in the experiment. The results are shown in Table 13:

Table 13. β-galactosidase expression in deletion derivatives of the 9.7 kb *ClaI-EcoRI* fragment of p170

	Miller units in GM17 (final pH 5.6-5.8)	Miller units in ArgM17 (final pH 6.6-6.8)	Miller Units in GM17 vs ArgM17
Integrant 170	1.7	0.1	17
L. lactis MG1363 containing plasmid			
pSMA339	15	1	15
pSMA340	16	1	16
pSMA341	7	7	1.0
pSMA342	2.1	1.5	1.4
pSMA343	22	8	2.8
pSMA344	14	1	14

Lactococcus lactis containing pSMA339, pSMA340 or pSMA344, show the same regulated expression of β-galactosidase as Integrant 170. This shows that the promoter P170 is regulated also when located on the a multicopy plasmid like pAK80. In contrast, the promoter carried on pSMA342 does not show a regulated expression. The promoter harboured on pSMA343 is regulated by pH or arginine. This regulation was not detected

in the plate assay. This might be due to differences in the growth on plates and in liquid medium. The regulation observed on the promoter harboured on pSMA343 is not as tight as the regulation of P170.

Fine mapping of the promoter P170 located on the 4 kb *ClaI-NdeI* fragment of p170.

Prior to fine mapping of P170 located on the 4 kb ClaI-NdeI fragment of p170, a more detailed restriction map of the 4 kb *ClaI-NdeI* fragment was produced (Figure 17).

The 4 kb *ClaI-NdeI* lactococcal fragment of p170 is harboured on pSMA202. pSMA202 contains three *HindIII* sites, of which two are located within the lactococcal DNA and one in the polylinker region. Insertion into pAK80 of the 1.3 kb *HindIII* fragment, extending from the *HindIII* site in the polylinker to the *HindIII* site in the *Lactococcus* DNA resulted in the plasmid, pSMA357. The insert in pSMA357 contained no promoter activity when introduced into *Lactococcus lactis* MG1363.

The 2.3 kb. *HindIII* fragment on the 4 kb *ClaI-NdeI* fragment was cloned into pAK80 digested with *HindIII*.The resulting plasmid, pSMA348, was introduced into *Lactococcus lactis* MG1363. From this plasmid β-galactosidase was expressed, which demonstrates the existence of a functional promoter within this *HindIII* fragment. A 1.5 kb *HincII* fragment was inserted into the *SmaI* site of pAK80 and the resulting plasmid, pSMA358, was introduced into *Lactococcus lactis* MG1363. β-galactosidase was expressed from pSMA358. The 1.5 kb *HincII* fragment covers most of the 1.3 kb *HindIII* fragment and has a 400 bp overlap with the adjacent 2.3 kb *HindIII* fragment. Based on promoter activity assessments on the inserts in the plasmids pSMA348, pSMA357 and pSMA358, the promoter P170 was mapped to a 400 bp *HincII-HindIII* fragment located about 1.3 kb upstream of Tn*917*-LTV1 insertion in Integrant 170.

(iv) Mapping of the promoter PSB.

From the sequencing of the upstream located DNA of SB a consensus promoter was identified [see Example 12 (i)] within a 190 bp *Hpa*I-*Cla*I fragment. pSB was digested with *Hpa*I and *Cla*I and the fragment was ligated to pNZ336 (Simons et al. 1990) digested with *Hpa*I and *Cla*I. The resulting plasmid, pNZ336:SB, was digested with *Sal*I and *Bam*HI. The 190 bp fragment was ligated to pAK80, digested with *Xho*I and *Bam*HI. The ligation mixture was introduced into *E. coli* DH5α, and the resulting plasmid, pSMA347 was subsequently introduced into *Lactococcus lactis* MG1363. Strain MG1363/pSMA347 expresses β-galactosidase, which demonstrate the existence of a functional promoter on the 190 bp fragment.

(v) Measurements on induced and non-induced overnight cultures of *Lactococcus lactis* MG1363 containing promoter harbouring pAK80 derivatives.

In Table 14, β-galactosidase activities on overnight cultures grown under induced and non-induced conditions, respectively, are given. The different growth conditions are temperature variations and variation of pH/concentration of arginine in the growth medium, respectively. The strains analyzed include both pAK80 derivatives containing *Eco*RI-*Cla*I fragments from the rescue plasmids and, based on the above mapping analyses, pAK80 derivatives containing deletions of the *Eco*RI-*Cla*I fragments. The growth of cultures as well as the β-galactosidase assay were performed as described in Example 11. In this example 5Arg1.5M17 is designated as 5ArgM17.

Table 14a. β-Galactosidase activities in overnight cultures grown at induced and non-induced conditions. Expression controlled by arginine and/or medium pH (30°C)

L. lactis containing plasmid	MEDIUM GM17 (final pH 5.6-5.8)	ArgM17 (final pH 6.6-6.8)	5ArgM17 (final pH 7.7-7.8)
pSMA332	680	560	
pSMA347	720	620	
Integrant SB:	6	18	
pSMA338	70	100	260
Integrant 162	18	51	140
pSMA339	15	1	0.4
pSMA340	16	1	0.7
pSMA344	14	1	0.5
Integrant 170	1.7	0.05	0.08

Tabel 14b. β-Galactosidase activities in overnight cultures grown at induced and non-induced conditions. Expression controlled by temperature (G1.5M17 medium)

PLASMID	30°C, 20 hrs	15°C, 165 hrs
pSMA337	190	35
Integrant 143	0.8	1.5
pSMA339	27	67
pSMA344	21	75
Integrant 170	1.7	14
pSMA347	650	120
Integrant SB	6	18
pSMA345	36	1.4
Integrant 172	1.4	0.9

The results show that the promoter from pSB is not pH regulated when harboured on pAK80. This result is seen with both

pSMA332 and pSMA347. The temperature regulation of the promoter from pSB is reversed when located on pAK80. The promoter from p162 is still regulated when located on pAK80. However, the total expression of β-galactosidase from the plasmid harboured promoter is not as high as expected from the high copy number of pAK80. The pH regulation of P170 is described above. The temperature regulation of P170 is conserved, although to a lesser extent, when located on pAK80. The promoter from p143 is regulated when located on pAK80. However, this regulation is opposite to the regulation observed when the promoter is chromosomally located. The strength of the promoter on p143 is increased dramatically when plasmid located. β-galactosidase expression from the promoter on p172 is slightly influenced by temperature when located on the chromosome. This regulation becomes much more pronounced when the promoter is plasmid located.

The results clearly demonstrate that regulation of a chromosomal promoter is in general dependent on the location, i.e. whether it is chromosomally or multicopy extrachromosomally located. It is contemplated that had a conventional promoter cloning strategy including shotgun cloning in a promoter cloning vector been used, the results concerning regulation would in most cases have been quite different from those obtained using the above strategy which included studies on regulation directly on chromosomally located promoters.

EXAMPLE 14

<u>The construction of a vector, pSMA500 that does not replicate in *Lactococcus lactis*</u>

For several microorganisms including *Lactococcus* it has been shown that a non-replicating vector can integrate into the chromosome, if the vector carries homologous DNA (Leenhouts et al. 1989). The integration mechanism involved is a single

cross-over event (Campbell-like integration) between the homologous DNA contained on the vector and on the chromosome. The result of this Campbell-like integration is a duplicate set of the homologous DNA on the chromosome and in between the duplicate set of homologous DNA, the non-replicating vector is located.

In contrast to Tn*917* insertion this Campbell-like integration results in a non destructive insertion, if an appropriate integratable vector is used.

A non-replicating vector, pSMA500, was constructed based on the *E. coli* plasmid pVA891 (Macrina et al. 1983) carrying an erythromycin resistance marker, and, as a reporter gene, the promoterless β-galactosidase genes derived from *Leuconostoc mesenteroides* subsp. *cremoris*.

The polylinker and the promoterless β-galactosidase genes from the plasmid pAK80 was cloned into the plasmid pVA891, which is unable to replicate in lactic acid bacteria. pAK80 was digested with *Hin*dIII and *Sal*I. The 4.1 kb fragment containing the polylinker and the β-galactosidase genes was purified and ligated to pVA891 also digested with *Hin*dIII and *Sal*I. This ligation mixture was introduced into *E. coli* MC1000, selecting for erythromycin resistance (Em^r) (250 μg/ml). The resulting plasmid was designated pSMA500. This vector is not able to replicate in lactic acid bacteria. However, if the plasmid is inserted into the bacterial chromosome, the erythromycin resistance gene is expressed in most lactic acid bacteria. When a functional promoter is cloned into the polylinker of pSMA500 the host bacterium will additionally express the β-galactosidase genes.

EXAMPLE 15

Insertion of a regulated promoter into pSMA500 and integration into the *Lactococcus* chromosome

(i) Insertion of promoters into pSMA500

The regulation of the promoters from p170 and pSB has been described in Example 8. In the present Example *Lactococcus* DNA from p170 containing the regulated promoter, P170 was inserted into pSMA500 and this construct subsequently integrated into the chromosome of *Lactococcus lactis* MG1363. In parallel, Lactococcus DNA from pSB, containing the regulatable promoter PSB, was inserted into pSMA500 and this construct subsequently integrated into the chromosome of *Lactococcus lactis* MG1614.

This experiment was performed to examine if a regulatable promoter and the β-galactosidase gene inserted into the chromosome via Campbell-like integration still would exhibit regulated expression of β-galactosidase.

(ii) Construction of the integrable vectors pSMA501 and pSMA502.

pSMA212 as described in Example 13, contains a 9.7 kb *Xho*I-*Bam*HI fragment. This fragment is essentially the same as the 9.7 kb *Lactococcus* DNA segment of p170, which harbours the regulated promoter P170. The 9.7 kb fragment from pSMA212 was cloned into pSMA500 also digested with *Xho*I and *Bam*HI. The resulting plasmid, pSMA501, was introduced into *E. coli* MC1000 and transformants selected for Em^r (250 μg/ml).

In parallel, the 1.8 kb *Xho*I-*Bam*HI *Lactococcus* DNA fragment from pGEM:SB (see Example 8), which harbours the regulated promoter PSB, was cloned into pSMA500. The resulting plasmid, pSMA502 was introduced into *E. coli* MC1000 and transformants

selected for Em^r (250 µg/ml). Standard DNA manipulations and transformations were according to Maniatis et al. 1982.

(iii) Integration of pSMA501 and pSMA502 into the *Lactococcus* chromosome.

About 2 µg Qiagen (Qiagen Plasmid Kit, Diagen, Düsseldorf, Germany) purified DNA of pSMA501 was introduced into *Lactococcus lactis* MG1363. In parallel, about 2 µg Qiagen purified DNA of pSMA502 was introduced into *Lactococcus lactis* MG1614. Transformation of *Lactococcus lactis* was as described in Example 1. The transformants were plated on SGM17 plates containing 1 µg/ml Em and 160 µg/ml X-gal. After growth at 30°C for 48 hours, only blue transformants appeared on both parallel set of plates. These results indicated that pSMA501 had integrated into the chromosome of strain MG1363 and that pSMA502 has integrated into the chromosome of strain MG1614. Also, the results showed that the promoters on pSMA501 and pSMA502, respectively, were functional when integrated into the chromosome. About 5000 colony forming units/µg DNA was obtained using the pSMA501 construction and about 500 CFU/µg DNA was obtained using pSMA502. Using the replicating plasmid pAK80 the transformation efficiency was 1 x 10EE7 CFU/µg in both strains. Transformation of strain MG1363 and strain MG1614 with pSMA500 showed less than 5 CFU/µg DNA, which clearly demonstrated that the integration of pSMA501 and pSMA502 was mediated by the chromosomal *Lactococcus* insert on these vectors. Ten primary, randomly picked transformants from each parallel set of plates were streaked on GM17 plates containing 1 µg/ml Em and 160 µg/ml X-gal. All colonies appearing after this streaking were homogeneous and blue. Plasmid DNA extractions from transformants revealed no detectable extrachromosomally plasmid DNA in the bacterial cell. This strongly indicated that the plasmids pSMA501 and pSMA502 had become integrated into the chromosome of the recipient strains. In Figure 18 is illustrated the Campbell-like integration of the non-replicating plasmids.

In order to study the stability of the integrated plasmids, both types of integrants were grown in the absence of Em selection for about 20 generations. Suitable dilutions of the resulting culture were plated on GM17 plates with X-gal and subsequently replicated to selective plates, GM17 + X-gal + 1μg/ml Em. In this plate assay no loss of β-galactosidase activity and Em resistance was detected.

(iv) Analysis of regulated β-galactosidase expression on *Lactococcus* strains harbouring integrable vectors on the chromosome.

The following experiments was performed to analyze if the expression of β-galactosidase is regulated in strain MG1363 harbouring chromosomally integrated pSMA501 (strain MG1363::pSMA501) and strain MG1614 harbouring chromosomally integrated pSMA502 (strain MG1614::pSMA502).

Six randomly picked reisolates of strain MG1363::pSMA501 were streaked on GM17 plates (1.2xM17-agar and 0.5 % glucose) and on ArgM17 plates (1.2xM17-agar, 0.1% glucose and 0.1% arginine). Both types of plates contained 1 μg/ml Em and 160 μg/ml X-gal. Isolates No. 6, 9, 10, 14 and 21 were all blue on GM17 plates and white on ArgM17 plates. This result shows that the β-galactosidase expression in these isolates, like in Integrant 170 (see Example 7), are still regulated in a pH dependent manner. Isolate No. 3 was blue on GM17 plates and pale blue on ArgM17 plates. The higher level of β-galactosidase expression of this isolate on both types of plates is possibly a consequence of the integration of several copies of the integrable vector into the chromosome or of an amplification of the non-tandem repeated chromosomal DNA sequence.

Eight randomly picked reisolates of strain MG1614::pSMA502 were streaked on GM17 plates and on ArgM17 plates. Both types of plates contained 1 μg/ml Em and 160 μg/ml X-gal. All isolates of strain MG1614::pSMA502, i.e.isolates no. 7, 8, 10, 13, 14, 17, 18, and 22 were blue on GM17 plates and

slightly more blue on ArgM17 plates. This result indicated at least a certain level of pH dependent β-galactosidase expression in the strain MG1614::pSMA502. However, in this plate assay it was not possible to compare the levels of β-galactosidase expression and hence the tightness of regulation in strain MG1614::pSMA502 and Integrant SB.

In Examples 7 and 11, the media consisting of 1.5 x M17 supplemented with 0.5% glucose and 1.5 x M17 supplemented with 0.1% glucose and 0.1% arginine were referred to as G1.5M17 and Arg1.5M17, respectively. In the following these media are designated GM17 and ArgM17, respectively.

The activity of β-galactosidase were measured in cultures grown for 17-18 hrs at 30°C in GM17 medium (pH 5.6 after growth) and in ArgM17 medium (pH 6.7 after growth), respectively. Both GM17 medium and ArgM17 medium contained 1 μg/ml erythromycin. Three reisolates of strain MG1363::pSMA501 and two reisolates of strain MG1614::pSMA502 were each assayed for β-galactosidase activity. As a control of regulated β-galactosidase expression, the Integrants 170 and SB, respectively were included in the experiment. The results are shown in Tables 15a and 15b below:

Table 15a. β-galactosidase activity of MG1363::pSMA501

Strain	Miller units in GM17 medium	Miller units in ArgM17 medium	Ratio of Miller units in GM17 vs ArgM17
Integrant 170	1.9	0.1	19
MG1363::pSMA501, Isolate No. 3	23.0	1.2	19
MG1363::pSMA501, Isolate No. 6	7.0	0.3	23
MG1363::pSMA501, Isolate No. 21	2.6	0.2	13

Table 15b. β-galactosidase activity of MG1614::pSMA502

Strain	Miller units in GM17 medium	Miller units in ArgM17 medium	Ratio of Miller units in ArgM17 vs GM17
Integrant SB	6.4	20.0	3.1
MG1614::pSMA502, Isolate No. 8	77.0	120.0	1.6
MG1614::pSMA502, Isolate No. 14	64.0	99.0	1.5

It is clearly demonstrated that the expression of the β-galactosidase gene is regulated in all three isolates of strain MG1363::pSMA501. The regulation in each isolate is similar to the regulation observed in Integrant 170. The differences in β-galactosidase activity levels is possibly due to differences in the copy number of pSMA501 on the chromosome. It is, however, difficult to conclude from the results shown in Table 15b, whether there is a regulated or non-regulated β-galactosidase expression in the two isolates of MG1614::pSMA502.

EXAMPLE 16

Transformation of *Lactobacillus helveticus* with pTV32 AND pLTV1.

Each of the transposition vectors, pTV32 and pLTV1, was electroporated into *Lactobacillus helveticus* CNRZ32 according to the method described by Bhowmik et al. 1993. The vector pNZ18 (NIZO, BA Ede, The Netherlands), conferring Cm resistance to the host, was also introduced into strain CNZR32 as control of transformation efficiency.

After electroporation, the transformed cells were plated on MRS agar (Oxoid) containing 10mM CaCl2 and an antibiotic depending on the vector used for transformation. The anti-

biotic and the concentration used for selection of transformants are given in Table 16 below. Also given in Table 16 are the results from the transformations. A blank space in the Table indicates that this experiment was not performed.

Table 16. Transformation of pTV32 and pLTV1 into *Lactobacillus helveticus* CNRZ 32

	Transformants per μg plasmid			
	pTV32	pLTV1	pNZ18	no plasmid
Antibiotic, concentration				
Tetracycline, 20 μg/ml		0		0
Chloramphenicol, 10 μg/ml	0	0	0	0
Erythromycin, 10 μg/ml	130	140		0

10 pTV32 transformants and 10 pLTV1 transformants were streaked on MRS agar containing 10 μg/ml Em. A reisolated colony from each of the 20 transformants was inoculated in MRS broth (Oxoid) containing 5 μg/ml Em and plasmid extraction was performed according to O'Sullivan et al. 1993. The plasmid extraction preparations were digested with *Eco*RI and then subjected to an agarose gel electrophoresis analysis.

No plasmid DNA was detected in any of these plasmid extractions. As it appears from the above Table, 130 and 140 transformants, respectively were obtained per μg of plasmid DNA in which transformants erythromycin resistance was expressed the only conclusion which can be drawn from the fact that plasmid DNA was not detected in any of the tested transformants expressing the erythromycin resistance is that the DNA introduced into the transformants had become integrated in the *Lactobacillus helveticus* chromosome.

The above results therefore provides a strong indication that the above Tn*917* derivatives can be used in accordance with the invention also in *Lactobacillus* spp.

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 42, line 11.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet [X]

Name of depositary institution

DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

Address of depositary institution *(including postal code and country)*

Mascheroder Weg 1B
D-38124 Braunschweig
Germany

Date of deposit	Accession Number
21 December 1992	DSM 7361

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

As regards the respective Patent Offices of the respective designated states, the applicants request that a sample of the deposited microorganisms only be made available to an expert nominated by the requester until the date on which the patent is granted or the date on which the application has been refused or withdrawn or is deemed to be withdrawn.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
[X] This sheet was received with the international application	[ ] This sheet was received by the International Bureau on:
Authorized officer Anne-Grethe Henriksson Senior Clerk	Authorized officer

Form PCT/RO/134 (July 1992)

INDICATIONS RELATING TO DEPOSITED MICROORGANISMS
(PCT Rule 12*bis*)

Additional sheet

In addition to the microorganism indicated on page 110 of the description, the following microorganisms have been deposited with

DSM-Deutsche Sammlung von Mikroorganismen und Cellkulturen GmbH
Mascheroder Weg 1b, D-38124 Braunschweig, Germany

on the dates and under the accession numbers as stated below:

Accession number	Date of deposit	Description Page No.	Description Line No.
DSM 7360	21 December 1992	45	26
DSM 8496	27 August 1993	56	29
DSM 8858	22 December 1993	58	10
DSM 8495	27 August 1993	67	10
DSM 8497	27 August 1993	67	11
DSM 8498	27 August 1993	67	11
DSM 8499	27 August 1993	67	11
DSM 8500	27 August 1993	67	12
DSM 8859	22 December 1993	70	18
DSM 8834	22 December 1993	79	2
DSM 8835	22 December 1993	79	2
DSM 8836	22 December 1993	79	2
DSM 8837	22 December 1993	79	2
DSM 8838	22 December 1993	79	2
DSM 8839	22 December 1993	79	2
DSM 8840	22 December 1993	79	3
DSM 8841	22 December 1993	79	3
DSM 8842	22 December 1993	79	3
DSM 8843	22 December 1993	79	3
DSM 8844	22 December 1993	79	3
DSM 8845	22 December 1993	79	3
DSM 8846	22 December 1993	79	4
DSM 8847	22 December 1993	79	4
DSM 8848	22 December 1993	79	4
DSM 8849	22 December 1993	79	4
DSM 8850	22 December 1993	79	4
DSM 8851	22 December 1993	79	4
DSM 8852	22 December 1993	79	5
DSM 8853	22 December 1993	79	5
DSM 8854	22 December 1993	79	5
DSM 8855	22 December 1993	79	5
DSM 8856	22 December 1993	79	5
DSM 8857	22 December 1993	79	5

For all of the above-identified deposited microorganisms, the following additional indications apply:

As regards the respective Patent Offices of the respective designated states, the applicants request that a sample of the deposited microorganisms stated above only be made available to an expert nominated by the requester until the date on which the patent is granted or the date on which the application has been refused or withdrawn or is deemed to be withdrawn.

CLAIMS

1. A method of isolating a lactic acid bacterial DNA fragment comprising a promoter, the method comprising the steps of:

(i) selecting a DNA molecule replicating in a lactic acid bacterium, said molecule comprising (a) a transposable element comprising a promoterless structural gene as a promoter probe gene, (b) a detectable selective marker gene, and (c) an origin of replication which is functional in a lactic acid bacterium,

(ii) introducing the DNA molecule into a population of a lactic acid bacterium, followed by subjecting the population to conditions allowing transposition of the transposable element to occur,

(iii) selecting a cell of the lactic acid bacterial population in which the promoterless gene is expressed,
(iv) cloning said cell and isolating from the clone a DNA fragment comprising a lactic acid bacterial promoter being operably linked to the originally promoterless gene.

2. A method according to claim 1 which further comprises isolating from the DNA fragment isolated in step (iv) a lactic acid bacterial DNA subfragment comprising the promoter.

3. A method according to claim 1 wherein the transposable element is one which becomes at least quasi-randomly integrated into a lactic acid bacterial replicon.

4. A method according to claim 3 wherein the lactic acid bacterial replicon is the chromosome.

5. A method according to claim 1 wherein the transposable element is the transposon Tn*917*.

6. A method according to claim 1 wherein the DNA molecule of step (i) is a pTV plasmid.

7. A method according to claim 6 wherein the DNA molecule is selected from pTV32 and pLTV1.

8. A method according to claim 1 wherein the promoterless structural gene is selected from a gene coding for a gene product conferring antibiotic resistance, a gene coding for a gene product complementing an auxotrophic deficiency and a gene coding for an enzyme having a detectable end product.

9. A method according to claim 8 wherein the promoterless structural gene is a β-galactosidase-encoding gene.

10. A method according to claim 1 wherein the population of a lactic acid bacterium into which the DNA molecule is introduced are selected from *Lactococcus* spp., *Streptococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* spp., *Brevibacterium* spp., *Propionibacterium* spp. and *Bifidobacterium* spp.

11. A method according to claim 10 wherein the population is of a lactic acid bacterium selected from *Lactococcus lactis*.

12. A method according to claim 11 wherein the lactic acid bacterium is a *Lactococcus lactis* subspecies *lactis* strain selected from the strain MG1614 and strain MG1363.

13. A method according to claim 1 wherein the isolated promoter is a regulatable promoter.

14. A method according to claim 13 wherein the isolated regulatable promoter is regulatable by a factor selected from the pH, the growth temperature, a temperature shift eliciting the expression of heat chock genes, the composition of the growth medium including the ionic strength/NaCl content and the presence/absence of purine nucleotide precursors, and the

growth phase/growth rate of the lactic acid bacterium into which the promoter-comprising DNA molecule is introduced.

15. A method of constructing a recombinant lactic acid bacterium comprising the steps of:

(i) isolating in accordance with the method of claim 1 a DNA fragment comprising a regulatable lactic acid bacterial promoter,

(ii) inserting the isolated fragment comprising the promoter into a lactic acid bacterium upstream of a gene coding for a desired gene product, the inserted promoter thereby becoming operably linked to said gene.

16. A method of constructing a recombinant lactic acid bacterium comprising the steps of:

(i) isolating in accordance with the method of claim 1 a DNA fragment comprising a regulatable lactic acid bacterial promoter,

(ii) inserting into a lactic acid bacterium a gene coding for a desired gene product,

(iii) inserting the isolated fragment comprising the promoter into the lactic acid bacterium resulting from step (ii) upstream of the gene coding for a desired gene product, the inserted promoter thereby becoming operably linked to said gene.

17. A method according to claim 16 wherein the inserted gene coding for a desired gene product is a heterologous gene.

18. A method according to claim 16 wherein the inserted gene is derived from a lactic acid bacterium.

19. A method according to claim 16 wherein the gene coding for a desired gene product is inserted on the same DNA fragment as the isolated fragment comprising the promoter.

20. A method according to claim 15 or 16 wherein the isolated fragment comprising the promoter is inserted into the chromosome of a lactic acid bacterial bacterium.

21. A method according to claim 15 or 16 wherein the isolated fragment comprising the promoter is inserted extrachromosomally.

22. A method according to claim 15 or 16 wherein the isolated fragment comprising the promoter comprises a further DNA fragment whereby the isolated promoter becomes regulated by a stochastic event selected from recombinational excision of the promoter, recombinational excision of a gene coding for a product which is positively needed for the promoter function and recombinational excision of a regulatory DNA fragment inhibiting the function of the promoter.

23. A method according to claim 22 wherein the further fragment results in recombinational excision of a regulatory sequence inhibiting the function of the promoter.

24. A method of constructing a recombinant lactic acid bacterium comprising the steps of:

(i) selecting a DNA molecule replicating in a lactic acid bacterium, said molecule comprising a transposable element comprising a promoterless structural gene as a promoter probe gene, (b) a detectable selective marker gene, and (c) an origin of replication which is functional in a lactic acid bacterium,

(ii) introducing under conditions allowing transposition of the transposable element to occur, the DNA molecule of step (i) into a population of a lactic acid bacterium,

(iii) selecting a cell of the lactic acid bacterial population in which the promoterless structural gene is regulatably expressed as a result of being operably linked to a regulatable native promoter of the lactic acid bacterial cell,

(iv) identifying the site(s) in a replicon of the lactic acid bacterial cell of step (iii) into which the transposable element is integratable, and

(v) inserting into a non-integrant cell of the lactic acid bacterial population a gene coding for a desired gene product at a site in a replicon as identified in step (iv) or at a functionally equivalent site, whereby the gene becomes operably linked to said native lactic acid bacterial promoter,

the expression of the inserted gene hereby being altered as compared to the expression of the gene when operably linked to its native promoter.

25. A method according to claim 24 wherein the gene coding for a desired gene product is a heterologous gene.

26. A method according to claim 25 wherein the gene is derived from a lactic acid bacterium.

27. A method according to claim 24 wherein the DNA molecule of step (i) is transposed into the chromosome of the lactic acid bacterial bacterium.

28. A method according to claim 24 wherein the DNA molecule of step (i) is transposed into an extrachromosomal replicon.

29. A recombinant lactic acid bacterium comprising a gene coding for a desired gene product and operably linked thereto a lactic acid bacterial regulatable promoter not natively associated with the gene, the presence of said promoter

resulting in the expression of the gene being altered as compared to the expression of the gene when operably linked to its native promoter.

30. A bacterium according to claim 29 in which the gene coding for a desired gene product is expressed at a level which is at least 10% different from the level at which the gene is naturally expressed.

31. A lactic acid bacterium according to claim 30 in which the gene coding for a desired gene product is expressed at a level which is at least 25% different from the rate at which the gene is naturally expressed.

32. A bacterium according to claim 29 in which the gene coding for a desired gene product is a chromosomal gene.

33. A bacterium according to claim 29 in which the gene coding for a desired gene product is an extrachromosomal gene.

34. A bacterium according to claim 29 in which the gene coding for a desired gene product is a native gene.

35. A bacterium according to claim 29 in which the gene coding for a desired gene product is a heterologous gene.

36. A bacterium according to claim 35 in which the heterologous gene is derived from a lactic acid bacterium.

37. A bacterium according to claim 29 in which the inserted regulatable promoter is regulated by a factor selected from pH, the growth temperature, a temperature shift eliciting the expression of heat chock genes, the composition of the growth medium including the ionic strength/NaCl content and the presence/absence of purine nucleotide precursors, and the growth phase/growth rate of the bacterium.

38. A bacterium according to claim 29 in which the isolated sequence comprising the promoter comprises a further sequence whereby the promoter becomes regulated by a stochastic event.

39. A bacterium according to claim 38 in which the further sequence results in recombinational excision of a regulatory sequence inhibiting the function of the promoter.

40. A bacterium according to claim 29 in which the promoter is located on a plasmid having run-away behaviour.

41. A bacterium according to claim 29 which is one selected from *Lactococcus* spp., *Streptococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* spp., *Brevibacterium* spp., *Propionibacterium* spp. and *Bifidobacterium* spp.

42. A bacterium according to claim 32 in which the promoter is derived from *Lactococcus* spp., *Streptococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* spp., *Brevibacterium* spp., *Propionibacterium* spp. and *Bifidobacterium* spp.

43. A bacterium according to claim 42 wherein the promoter is derived from *Lactococcus lactis*.

44. A bacterium according to claim 43 wherein the promoter is a promoter selected from a promoter isolated from strain MG1614 and strain MG1363.

45. A bacterium according to claim 29 in which a gene coding for a desired gene product is inserted at a site in a replicon where it is under the control of a promoter present in the replicon, which site is identifiable by the insertion of a promoterless structural gene by means of a transposable element comprising the promoterless structural gene whereby the originally promoterless gene becomes expressible by being operably linked to the promoter present in said replicon, the insertion of the gene at said site having resulted in said

gene becoming operably linked to the promoter being present in the replicon.

46. A bacterium according to claim 45 wherein the inserted gene is derived from a lactic acid bacterium.

47. A bacterium according to claim 45 wherein the gene is inserted into the chromosome of the lactic acid bacterial bacterium.

48. A bacterium according to claim 45 wherein the gene is inserted into an extrachromosomal replicon.

49. A bacterium according to claim 45 in which the inserted gene is a homologous gene.

50. A bacterium according to claim 45 in which the inserted gene is a heterologous gene.

51. A bacterium according to claim 50 in which the inserted heterologous gene is derived from a lactic acid bacterium.

52. A bacterium according to claim 29 or 45 in which the gene coding for a desired gene product is selected from a gene coding for a lipase, a gene coding for a peptidase, a gene coding for a protease, a gene coding for a gene product involved in carbohydrate metabolism, a gene coding for a gene product involved in citrate metabolism, a gene coding for a gene product involved in purine metabolism, a gene coding for a gene product involved in bacteriophage resistance, a gene coding for a lytic enzyme and a gene coding for a bacteriocin.

53. A bacterium according to claim 52 in which the gene coding for a desired gene product is selected from the *lacL* gene of a *Leuconostoc* spp, the *lacM* gene of a *Leuconostoc* spp. and a *Lactococcus lactis* ssp *lactis* gene coding for a lysine aminopeptidase.

54. An isolated DNA fragment comprising a regulatable lactic acid bacterial promoter which is functional in a lactic acid bacterium and operably linked thereto, a gene coding for a desired gene product, said promoter being one which is not naturally associated with the gene.

55. A DNA fragment according to claim 54 which further comprises at least one transcription terminator.

56. A DNA fragment according to claim 54 which is a DNA fragment having a size which is in the range of 100 to 10000 base pairs.

57. A DNA fragment according to claim 56 which is a fragment having a size which is in the range of 200 to 5000 base pairs.

58. A DNA fragment according to claim 54 which further comprises sequences coding for gene products involved in the regulation of the promoter.

59. A DNA fragment according to claim 54 in which the gene coding for a desired gene product is one selected from a gene coding for a lipase, a gene coding for a peptidase, a gene coding for a protease, a gene coding for a gene product involved in carbohydrate metabolism, a gene coding for a gene product involved in citrate metabolism, a gene coding for a gene product involved in purine metabolism, a gene coding for a gene product involved in bacteriophage resistance, a gene coding for a lytic enzyme and a gene coding for a bacteriocin.

60. A DNA fragment according to claim 54 in which the gene coding for a desired gene product is a heterologous gene.

61. A DNA fragment according to claim 54 in which the gene is a gene derived from a lactic acid bacterium.

62. A DNA fragment according to claim 61 in which the gene coding for a desired gene products is one selected from the *lacL* gene of a *Leuconostoc* spp., the *lacM* gene of a *Leuconostoc* spp. and a *Lactococcus lactis* ssp. *lactis* gene coding for a lysine aminopeptidase.

63. A DNA fragment according to claim 54 in which the lactic acid bacterial promoter is the regulatable promoter contained in the *Lactococcus lactis* ssp. *lactis* MG1363 integrant clone P139-170 deposited under the accession number DSM 7360.

64. A DNA fragment according to claim 54 in which the lactic acid bacterial promoter is the promoter contained in the *Lactococcus lactis* ssp. *lactis* MG1614 integrant clone 63b deposited under the accession number DSM 7361.

65. Use of a recombinant lactic acid bacterium as defined in claim 29 in the manufacturing of food products.

66. Use of a recombinant lactic acid bacterium as defined in claim 29 in the preservation of animal feed.

67. Use of a recombinant lactic acid bacterium as defined in claim 29 in the manufacturing of a probiotically active composition.

68. A bacterium according to claim 29 wherein the regulatable promoter is a promoter selected from a lactic acid bacterial tRNA promoter, rRNA promoter, *purD* promoter and a promoter comprising the motif AGTT.

69. A bacterium according to claim 29 in which the regulatable lactic acid bacterial promoter is inserted into a vector comprising a promoterless gene coding for a desired gene product, a theta-replicating lactic acid bacterial replicon which is functional in the bacterium, an insertion site allowing the DNA sequence to be inserted so that the

gene coding for the desired gene product is operably linked to the promoter, whereby the gene is transcribed.

70. A bacterium according to claim 69 in which the vector is the plasmid pAK80 as deposited under the accession number DSM 8496.

71. A recombinant plasmid comprising (i) a vector comprising a promoterless gene coding for a desired gene product, (ii) a theta-replicating lactic acid bacterial replicon which is functional in a lactic acid bacterium and (iii) an insertion site allowing a DNA sequence to be inserted, and (iv) inserted into said insertion site a DNA sequence comprising a regulatable lactic acid bacterial promoter, the insertion resulting in that the gene coding for the desired gene product is operably linked to the promoter, whereby the gene is transcribed.

72. A plasmid according to claim 71 in which the vector is pAK80.

73. A plasmid according to claim 71 wherein the regulatable promoter being inserted is a strong promoter selected from a lactic acid bacterial tRNA promoter, rRNA promoter, *purD* promoter and a promoter comprising the motif AGTT.

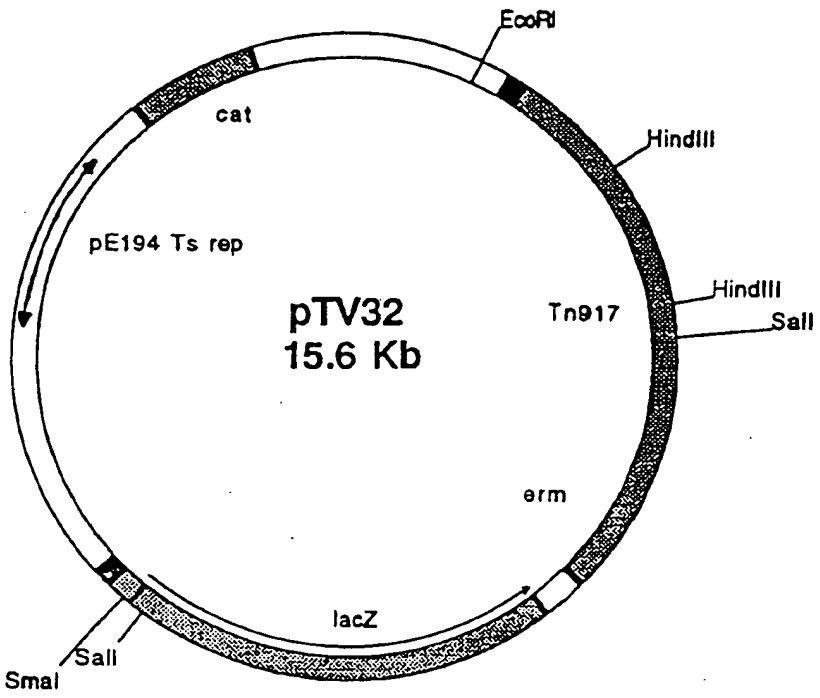
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Fig. 1

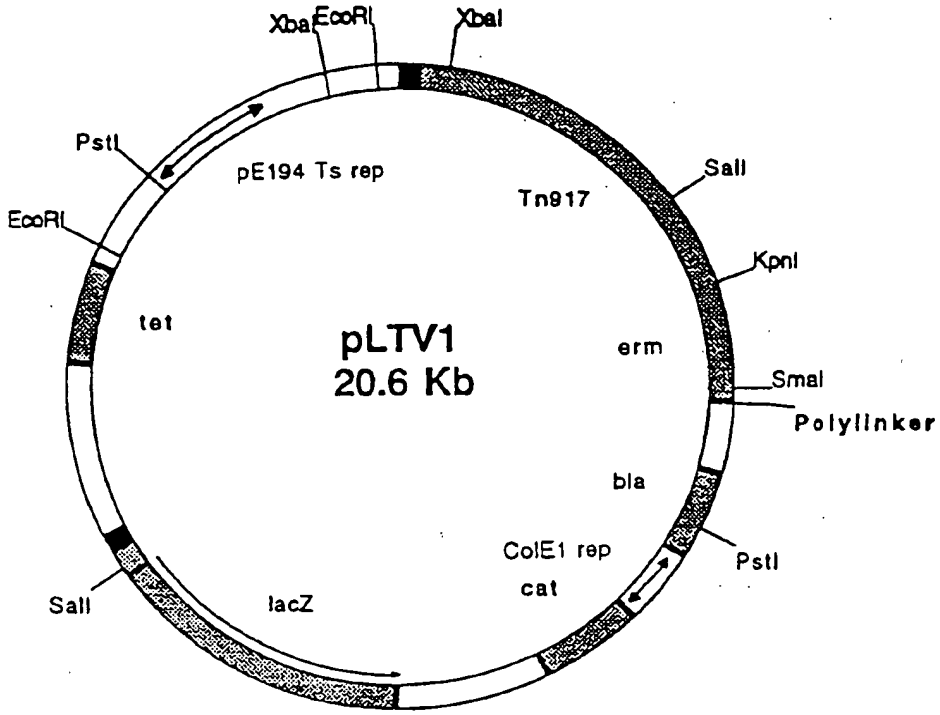
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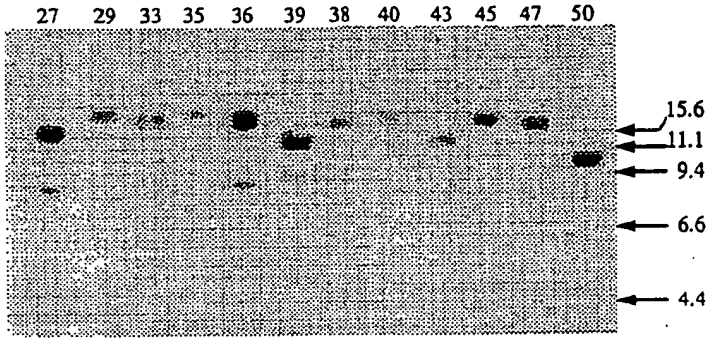
Fig. 2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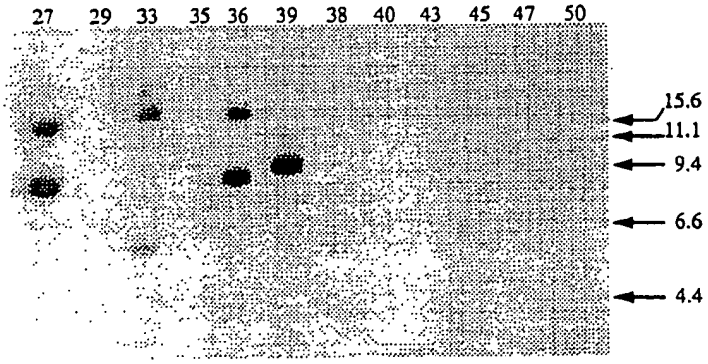
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Fig. 3B

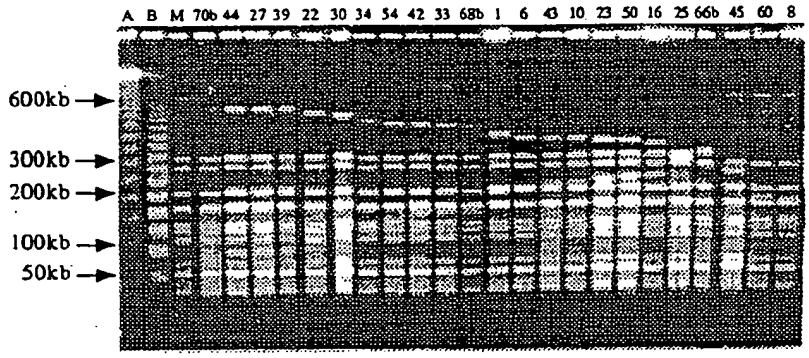
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Fig. 4A

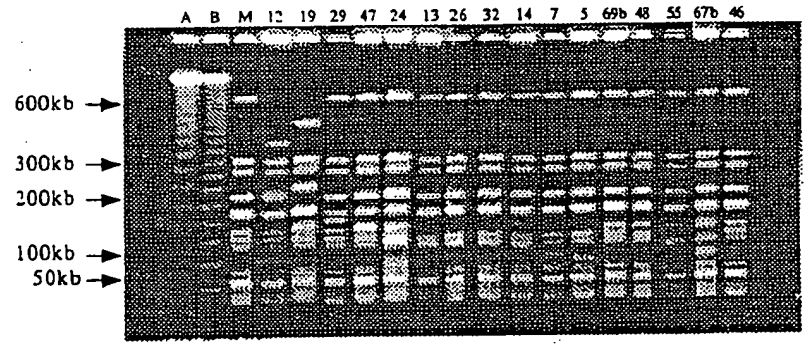
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Fig. 4B

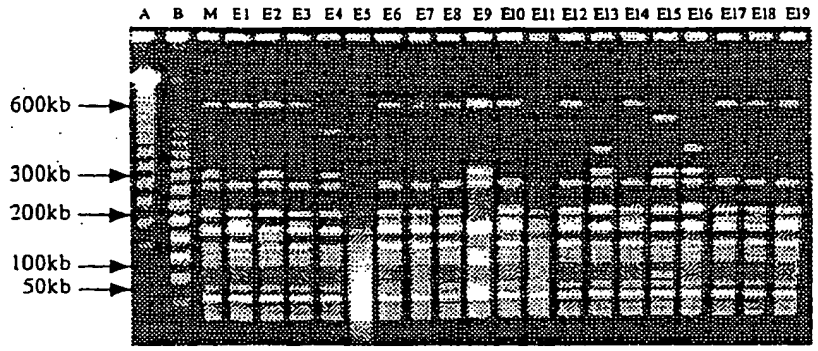
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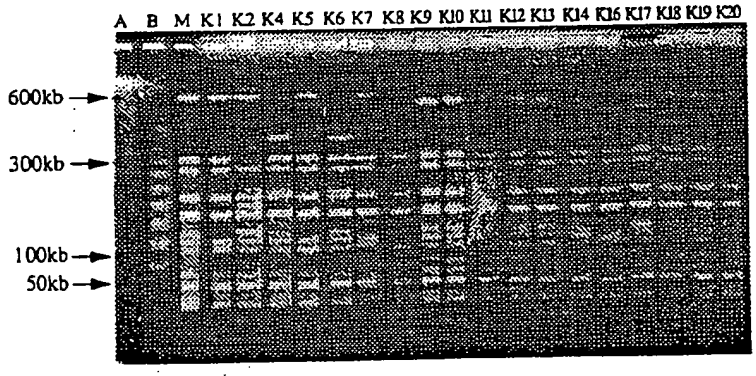
Fig. 5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300kb
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Fig. 6

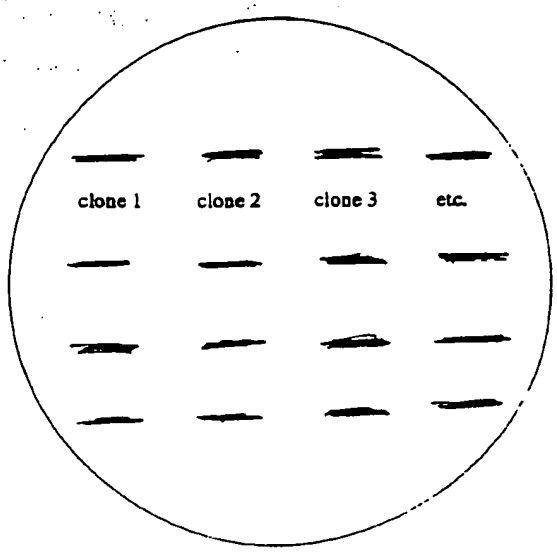
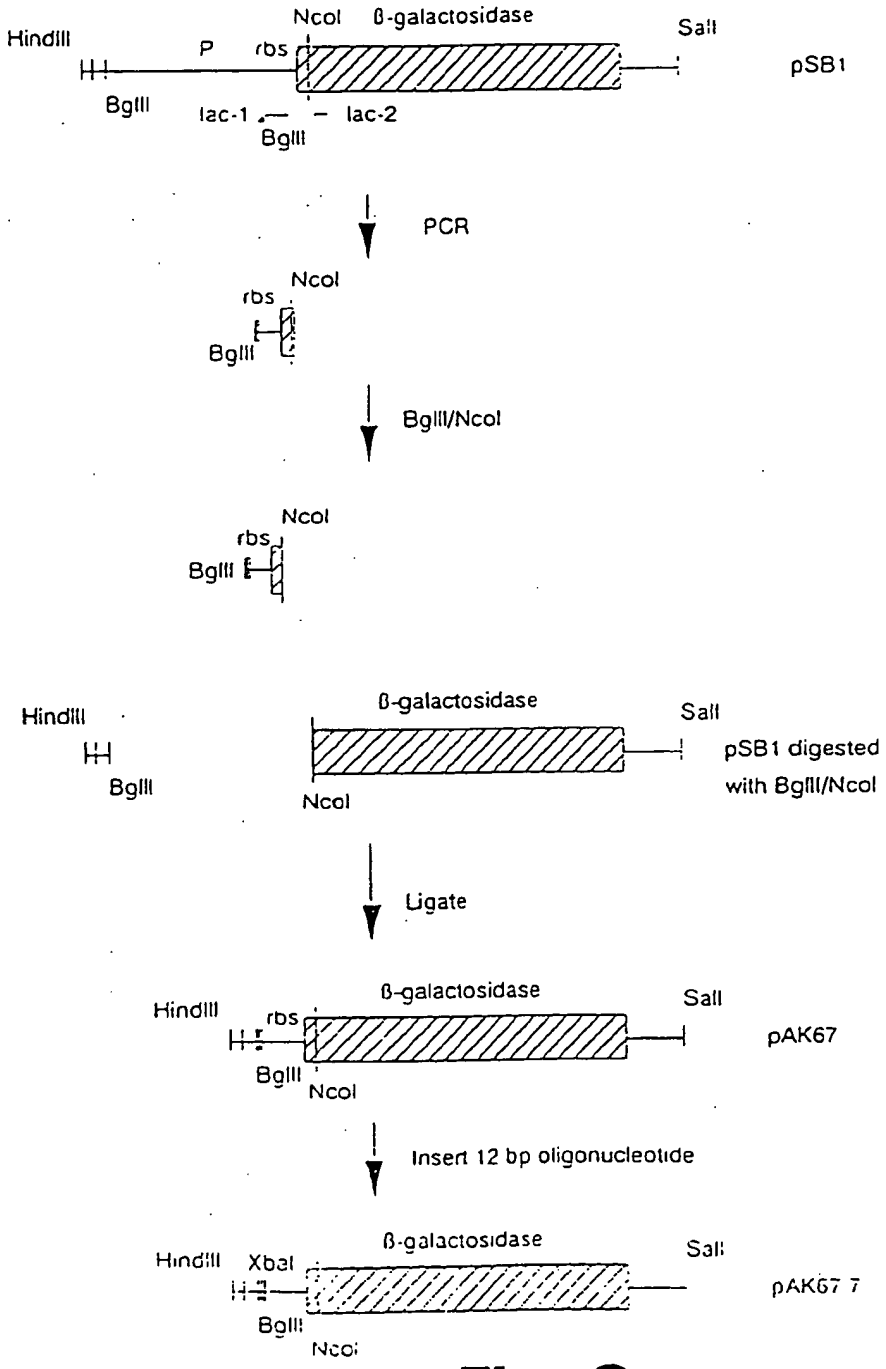


Fig. 7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BglII
BglII/NcoI
NcoI
rbs
BglII
HindIII
ß-galactosidase
SalI
pSB1 digested
with BglII/NcoI
BglII
NcoI
Ligate
HindIII
ß-galactosidase
SalI
rbs
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SalI
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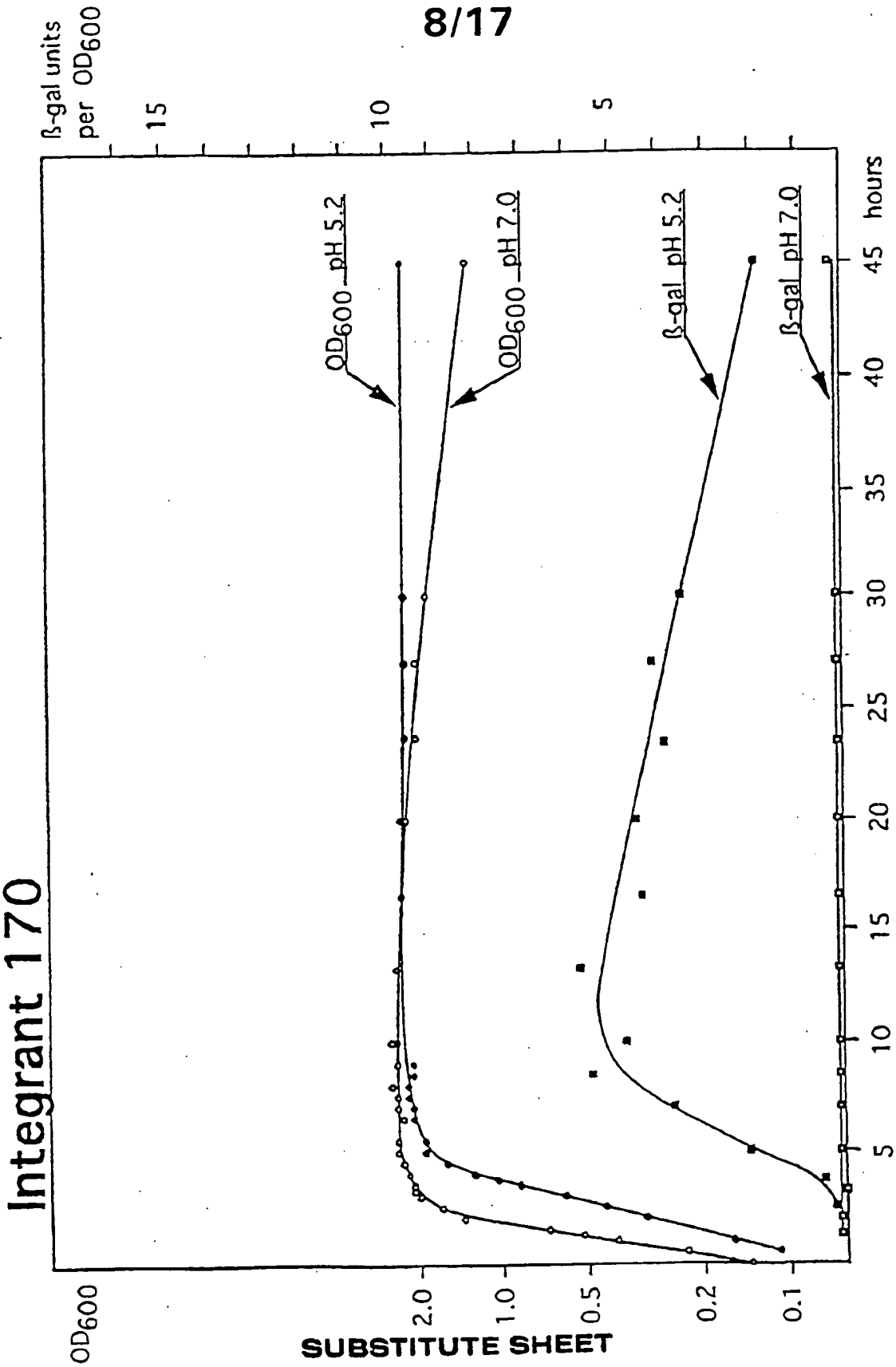
NcoI

Fig. 8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ß-gal units per OD_{600}
15
10
5
OD_{600} pH 5.2
OD_{600} pH 7.0
ß-gal pH 5.2
ß-gal pH 7.0
45 hours
40
35
30
25
20
15
10
5
OD_{600}
2.0
1.0
0.5
0.2
0.1

Fig. 9

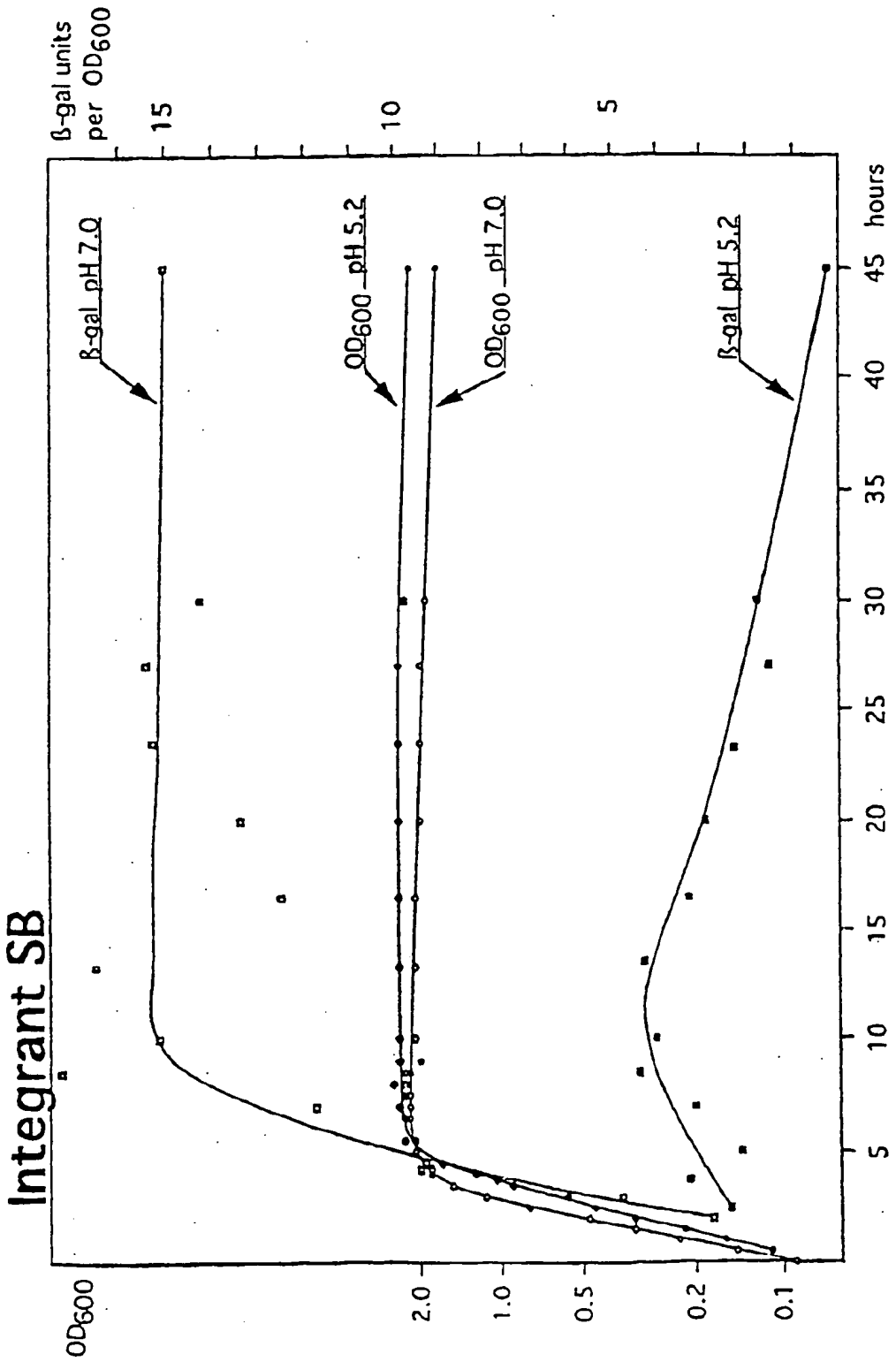
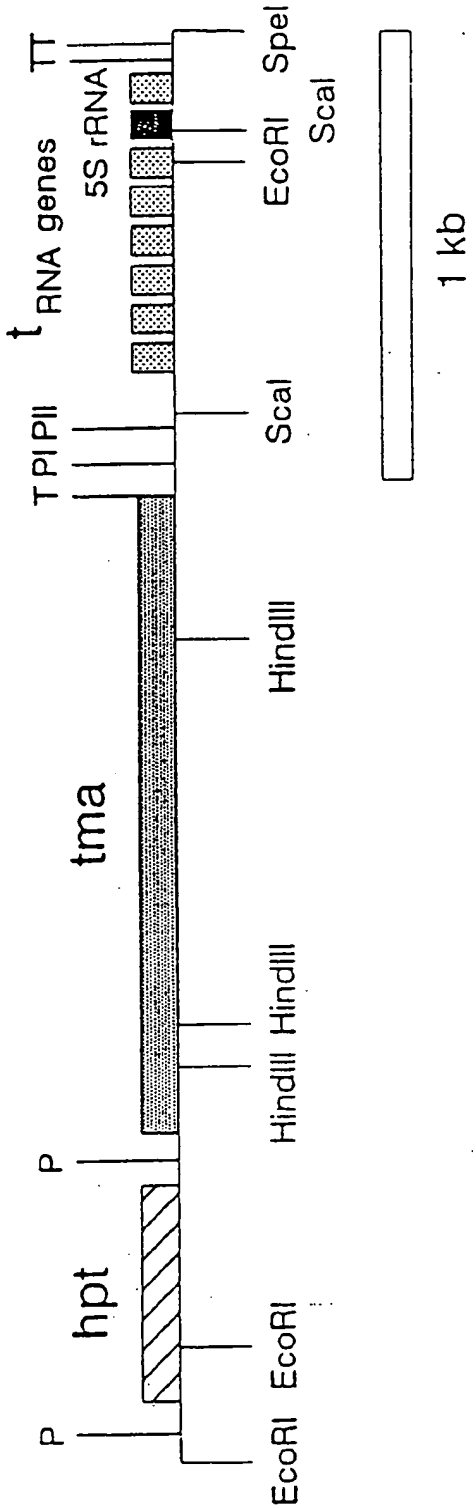

Integrant SB
ß-gal units per OD600
15
10
5
ß-gal pH 7.0
OD600 pH 5.2
OD600 pH 7.0
ß-gal pH 5.2
45 hours
40
35
30
25
20
15
10
5
OD600
2.0
1.0
0.5
0.2
0.1

Fig. 10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EcoRI
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EcoRI EcoRI
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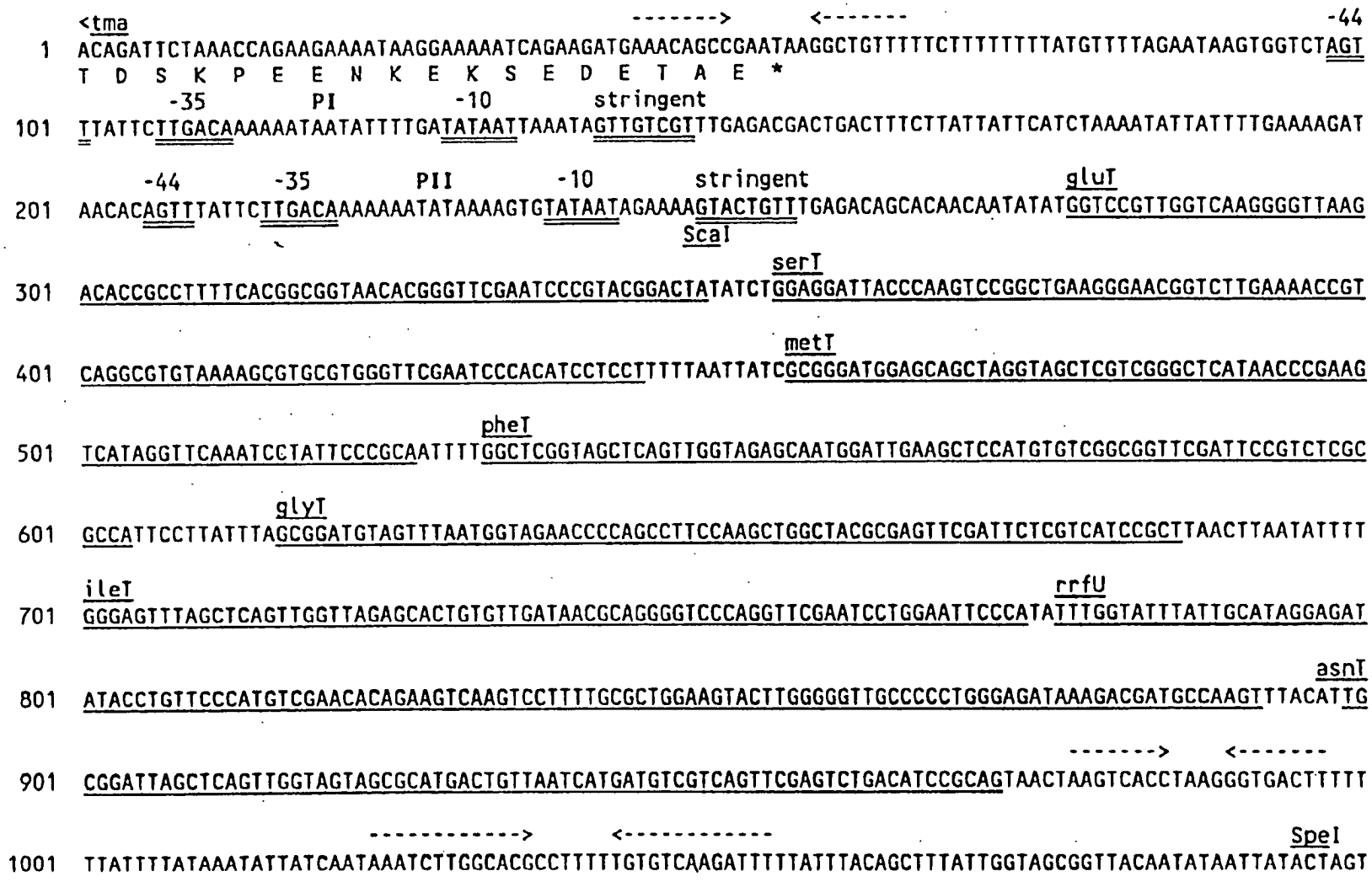
Fig. 11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T D S K P E E N K E K S E D E T A E *
-35 PI -10 stringent
101 TTATTCTTGACAAAAAATAATATTTTGATATAATTAAATAGTTGTCGTTTGAGACGACTGACTTTCTTATTATTCATCTAAAATATTATTTTGAAAAGAT
-44 -35 PII -10 stringent gluT
201 AACACAGTTTATTCTTGACAAAAAAATATAAAAGTGTATAATAGAAAAGTACTGTTTGAGACAGCACAACAATATATGGTCCGTTGGTCAAGGGGTTAAG
ScaI
serT
301 ACACCGCCTTTTCACGGCGGTAACACGGGTTCGAATCCCGTACGGACTATATCTGGAGGATTACCCAAGTCCGGCTGAAGGGAACGGTCTTGAAAACCGT
metT
401 CAGGCGTGTAAAAGCGTGCGTGGGTTCGAATCCCACATCCTCCTTTTTAATTATCGCGGGATGGAGCAGCTAGGTAGCTCGTCGGGCTCATAACCCGAAG
pheT
501 TCATAGGTTCAAATCCTATTCCCGCAATTTTGGCTCGGTAGCTCAGTTGGTAGAGCAATGGATTGAAGCTCCATGTGTCGGCGGTTCGATTCCGTCTCGC
glyT
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ileT rrfU
701 GGGAGTTTAGCTCAGTTGGTTAGAGCACTGTGTTGATAACGCAGGGGTCCCAGGTTCGAATCCTGGAATTCCCATATTTGGTATTTATTGCATAGGAGAT
asnT
801 ATACCTGTTCCCATGTCGAACACAGAAGTCAAGTCCTTTTGCGCTGGAAGTACTTGGGGGTTGCCCCCTGGGAGATAAAGACGATGCCAAGTTTACATTG
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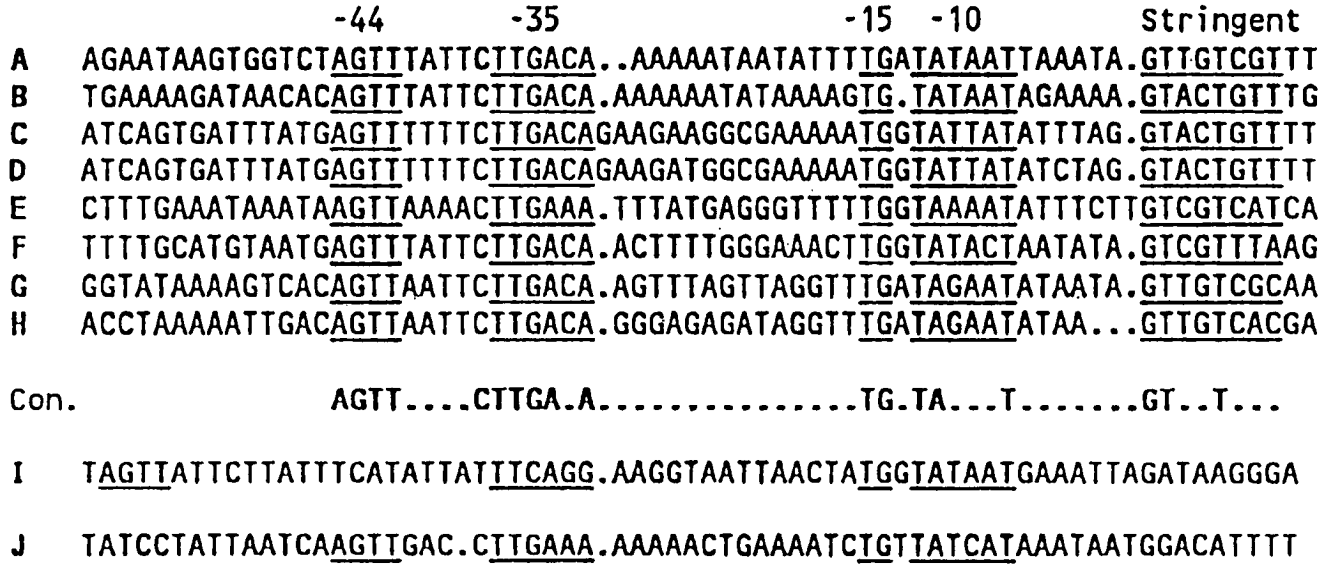
Fig. 12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C ATCAGTGATTTATGAGTTTTTTCTTGACAGAAGAAGGCGAAAAATGGTATTATATTTAG.GTACTGTTTT
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F TTTTGCATGTAATGAGTTTATTCTTGACA.ACTTTTGGGAAACTTGGTATACTAATATA.GTCGTTTAAG
G GGTATAAAAGTCACAGTTAATTCTTGACA.AGTTTAGTTAGGTTTGATAGAATATAATA.GTTGTCGCAA
H ACCTAAAAATTGACAGTTAATTCTTGACA.GGGAGAGATAGGTTTGATAGAATATAA...GTTGTCACGA
Con. AGTT....CTTGA.A.....TG.TA...T.....GT..T...
I TAGTTATTCTTATTTCATATTATTTCAGG.AAGGTAATTAACTATGGTATAATGAAATTAGATAAGGGA
J TATCCTATTAATCAAGTTGAC.CTTGAAA.AAAAACTGAAAATCTGTTATCATAAATAATGGACATTTT

Fig. 13

SUBSTITUTE SHEET


```
   1 GATCATCTACAATCATTAAAAGTTTATCAAAGAGCCGAAGATAGTTCTCAGATTCTGGTGGATAATCCATCATCAATTGTGCGAGGTTTGGATAAGATTC  100

 101 TAAAACAGTATAAATATTTTTACCATAGACGAAAAGGCGGTCTTGCCACGTTTTCTCATTTGCTGGAAAAATAATTTCAAGGCTTGCTTTATCAATAAGT  200

 201 TCTGCAAAAAGTGCTTCTTTACTTGAAAAATGGTAGTAAAGAGAAGACACTGTCATTCCTACGGCACTAGCTAACTTACGCATTGAAAATTCTGTGAGAG  300

 301 TAAGTTCTTCTAAAAGTTCCCAGCTGCTACTAATAATTTTATCTTTGGTTGGTTGAGTCGCCATAAGTTTTCGCTTTCTTTTTCTACTTAGATTTATTTT  400

 401 ACATGTTTTTAATGAAAATTGCGATAGAAAAGCTGATAAACAAATTTGTCATTTAAATATTGTAAGGGGAAAACTCTAGCTATAATTGAGTAAATACCGA  500

 501 ACAATCTCTCTTCTTATTTCTTGAAACTTTTGTTCAGGCTTTTTCTTTTATCACAAATCTTTTAAGATAGAATTATAAGATTTATAAAGCAAGAAAAGAT  600

 601 AGATGAGCTATCGTCACTTTGACTTTTATTATTCGTTCAAGATTTGTTGAATAATAAATAAAATAGCTGAATACACAAGTCTGTGTATATAAAAAGCGTT  700

                .RBS
 701 TGGGAATATCGGAGAAATGATGAAAATTTTGGTAATTGGTTCTGGCGGCCGCGAACATGCCTTAGCAAAAAAATTTATGGAAAGTCCTCAAGTTGAAGAA  800
                      M  K  I  L  V  I  G  S  G  G  R  E  H  A  L  A  K  K  F  M  E  S  P  Q  V  E  E
                      PurD-->

                                                .EcoRI
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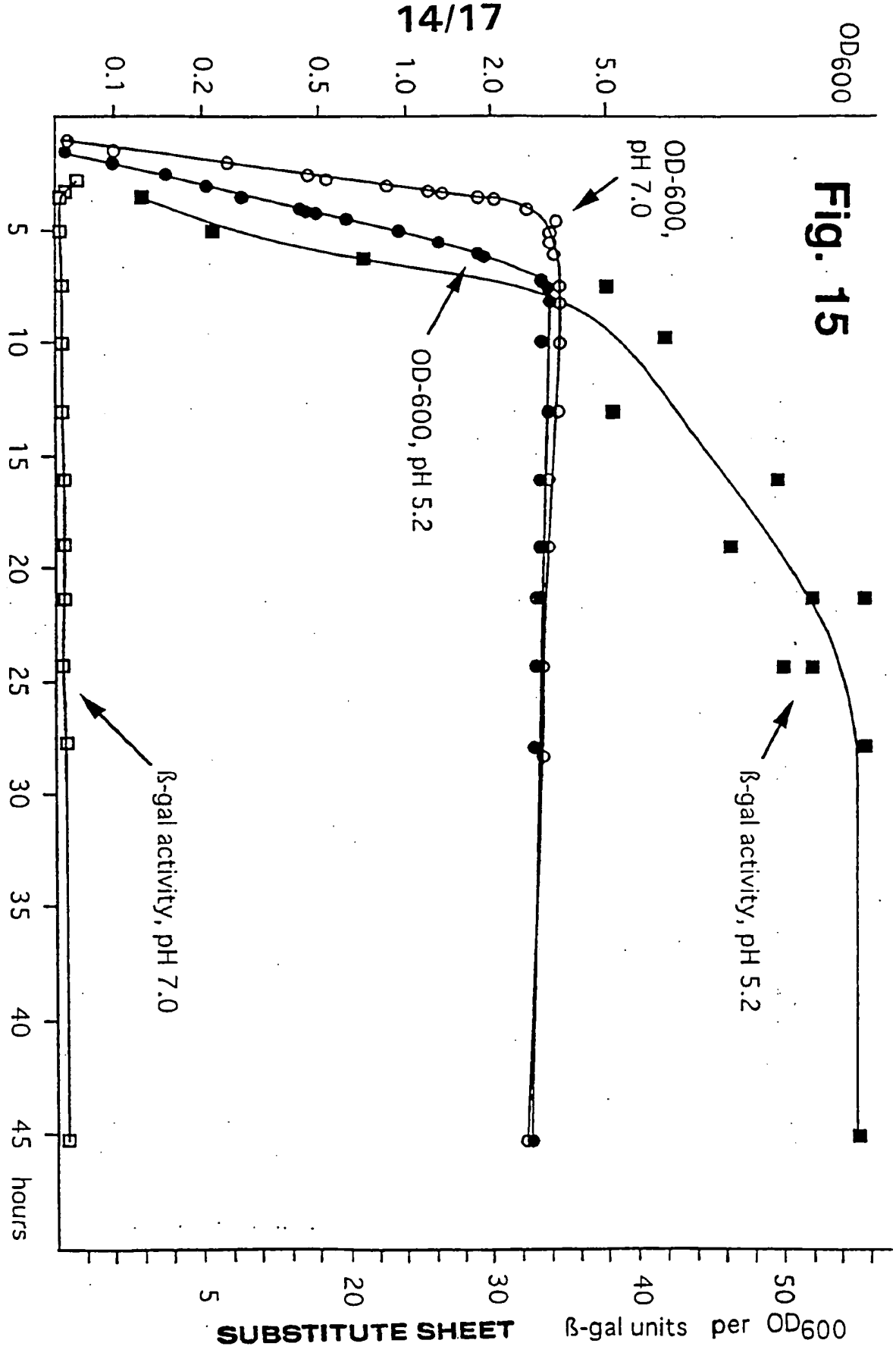
Fig. 14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5.0
OD-600, pH 7.0
OD-600, pH 5.2
ß-gal activity, pH 7.0
ß-gal activity, pH 5.2
5
10
15
20
25
30
35
40
45 hours
5
20
30
40
50
ß-gal units per OD600

Fig. 15

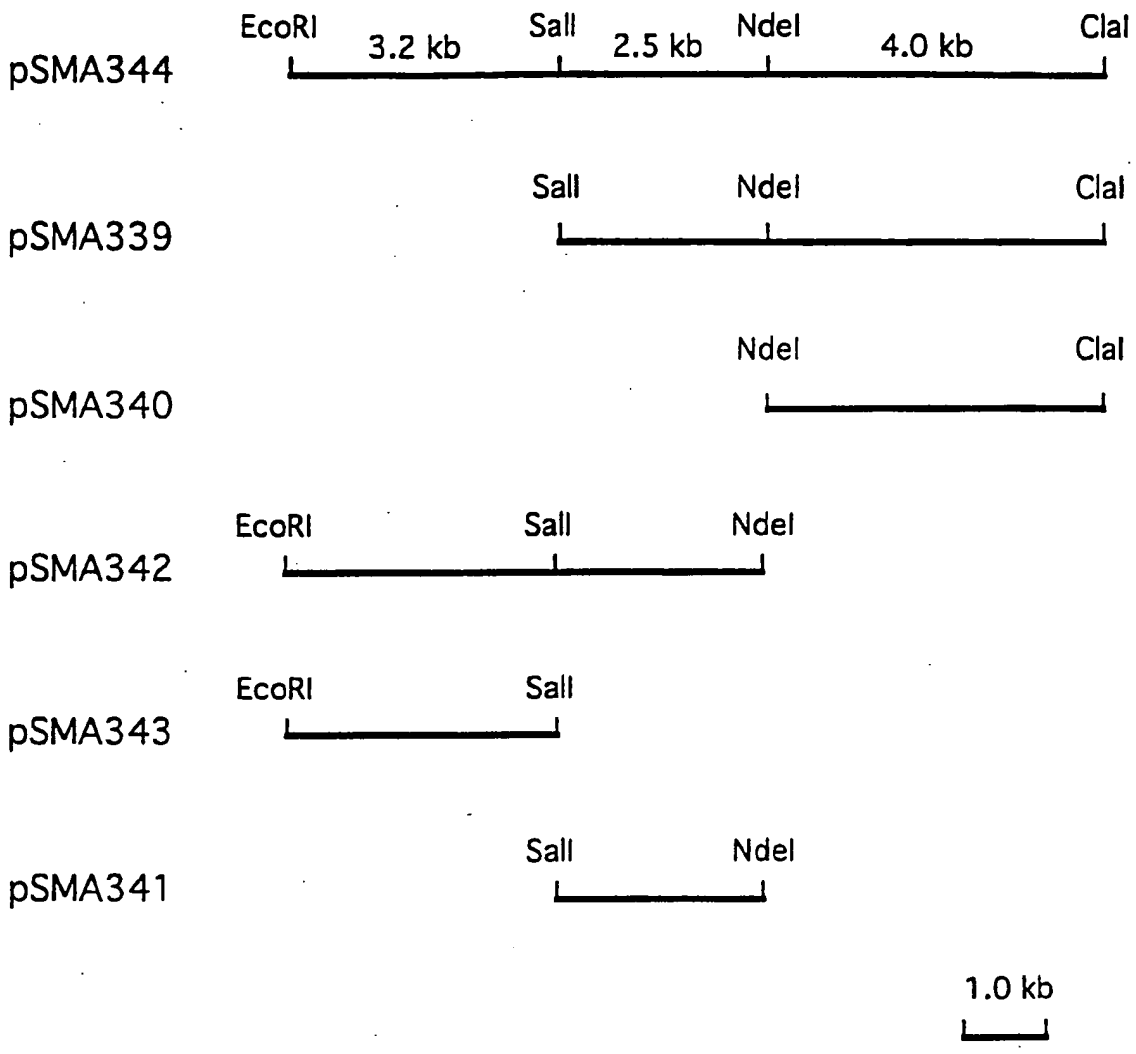
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Fig. 16

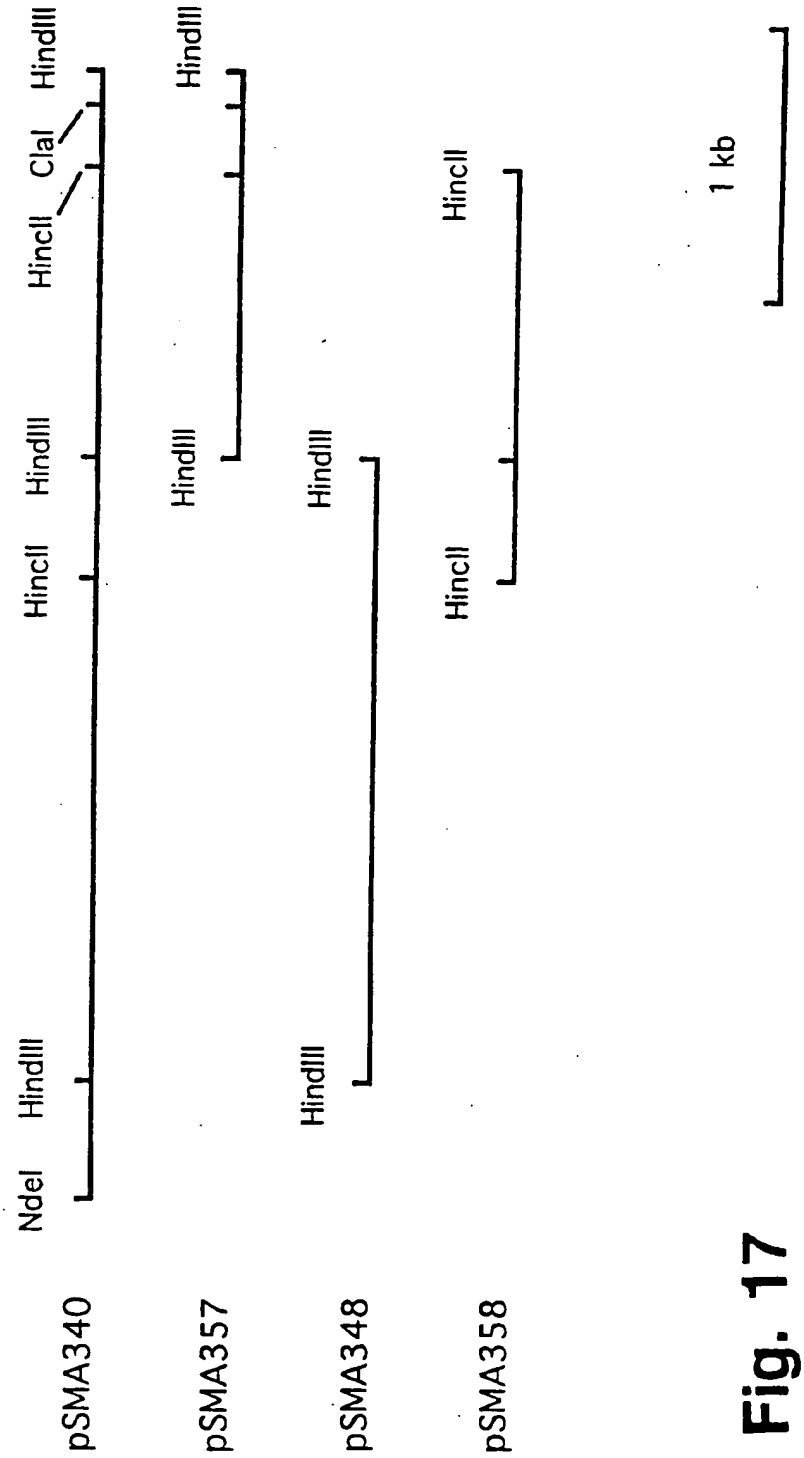
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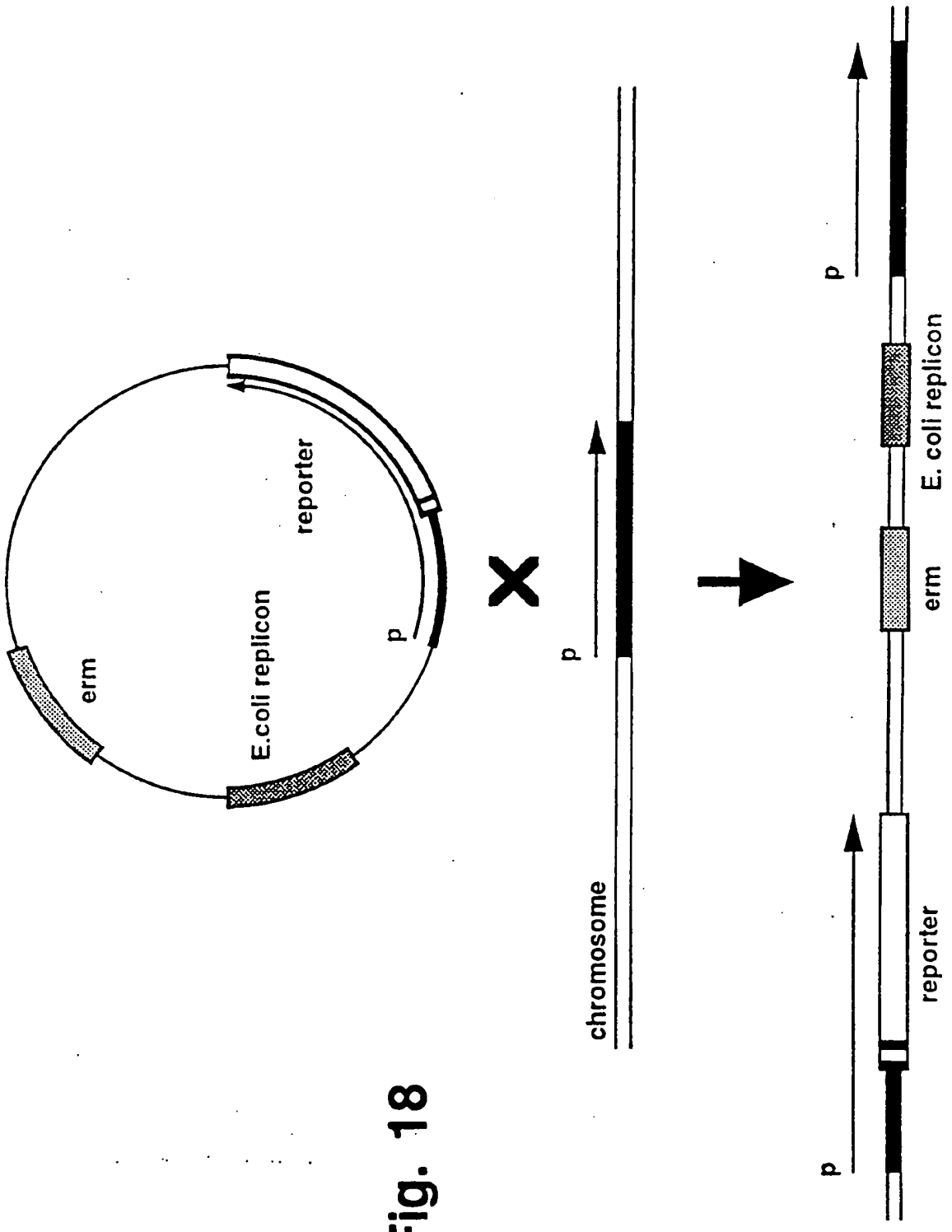
Fig. 17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E.coli replicon
erm
p
chromosome
p
p
E. coli replicon
erm
reporter
p

Fig. 18

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/DK 94/00004

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/74 C12N15/77 A23C9/12 A23L1/03

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N A23C A23L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	APPLIED AND ENVIRONMENTAL MICROBIOLOGY vol. 57, no. 2 , February 1991 pages 341 - 348 MERVI SIBAKOV ET AL. 'Secretion of TEM beta-lactamase with signal sequences isolated from the chromosome of Lactococcus lactis subsp. lactis' see abstract see page 341, left column, paragraph 2; table 1 see page 343, left column, paragraph 1 - right column, paragraph 3 see page 343, right column, last paragraph - page 344, left column, paragraph 1 see page 344, right column, paragraph 3 - page 345, right column, paragraph 1 --- -/--	1-4,8, 10-12, 15-20, 24-27, 29-32, 35,37, 41-45, 50-52, 54,59-61

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 March 1994	25 -04- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Montero Lopez, B

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 380 823 (BCZ FRIESLAND B.V.) 8 August 1990 see column 3, line 3 - column 4, line 30 see column 6, line 9 - line 57 see page 16, line 33 - page 18, line 14	29,32, 33,36, 38, 44-46, 57, 59-62, 64,65,70
X	EP,A,0 307 011 (NEDERLANDS INSTITUUT VOOR ZUIVELONDERZOEK) 15 March 1989 see column 2, line 7 - column 3, line 31 see column 11, line 40 - column 13, line 6 see column 15, line 33 - column 17, line 4	29,32, 33, 35-37, 44,45,57
Y	WO,A,92 04451 (GENESIT OY) 19 March 1992 see page 5, line 23 - page 8, line 12 see page 20, line 1 - line 9 see page 21, line 16 - line 24 see page 26, line 6 - page 37, line 25 see page 38, line 19 - page 40, line 3	1,2,8, 10-12, 15,16, 18,19, 24,26, 29,30,32
Y		33,35, 44-48, 51,54, 56-59, 64,66, 67,70
Y	EP,A,0 228 726 (STICHTING NEDERLANDS INSTITUUT VOOR ZUIVELONDERZOEK) 15 July 1987 see page 2, line 1 - line 16 see page 3, line 40 - page 5, line 32; examples V,VI	1,2, 8-10, 15-19, 24-26, 29,32, 59,70

-/--

1

INTERNATIONAL SEARCH REPORT

Inte: mal Application No
PCT/DK 94/00004

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	APPLIED AND ENVIRONMENTAL MICROBIOLOGY vol. 53, no. 10 , October 1987 pages 2452 - 2457 JOS M.B.M. VAN DER VOSSEN ET AL. 'Isolation and characterization of Streptococcus cremoris Wg2-specific promoters' see abstract see page 2452, left column, paragraph 2 see page 2453, right column, paragraph 2 - page 2454, left column, paragraph 1 see page 2454, right column, paragraph 4 - page 2456, left column, paragraph 1 see page 2456, right column, last paragraph - page 2457, left column, paragraph 2 ---	1,2,8, 10,13, 15,16, 19,59,64
Y	DEVELOPMENTS IN INDUSTRIAL MICROBIOLOGY vol. 31, no. 5 , 1990 pages 31 - 39 GUUS SIMONS ET AL. 'Construction of a promoter-probe vector for lactic acid bacteria using the lacG gene of Lactococcus lactis' see abstract see page 32, right column, paragraph 5 see page 35, left column, paragraph 3 - right column, paragraph 1 see page 36, left column, paragraph 1 - right column, paragraph 1 see page 37, left column, paragraph 2 ---	1,8-12
Y	APPLIED AND ENVIRONMENTAL MICROBIOLOGY vol. 53, no. 8 , August 1987 pages 1730 - 1736 M.E. SANDERS ET AL. 'A method for genetic transformation of nonprotoplasted Streptococcus lactis' see page 1730, left column, paragraph 3 - right column, paragraph 1 see page 1734, left column, paragraph 2 - page 1735, left column, paragraph 1 --- -/--	1,2, 8-13, 15-19, 24-26, 29,30, 32,33,35
Y		44-48, 51,54, 56-59, 64,66, 67,70

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/DK 94/00004

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	APPLIED AND ENVIRONMENTAL MICROBIOLOGY vol. 59, no. 1 , January 1993 pages 21 - 26 HANS ISRAELSEN ET AL. 'Insertion of transposon Tn917 into the Lactococcus lactis subsp. lactis chromosome' see abstract see page 21, left column, last paragraph - right column, paragraph 2; table 1 see page 22, right column, paragraph 4 see page 22, right column, paragraph 7 - page 23, right column, paragraph 1 see page 24, left column, paragraph 1 - right column, paragraph 1 see page 25, right column, paragraph 3 - page 26, left column, paragraph 2	1,3-14

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INTERNATIONAL SEARCH REPORT

...formation on patent family members

Inte nal Application No
PCT/DK 94/00004

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0380823	08-08-90	NL-A-	8803004	02-07-90
EP-A-0307011	15-03-89	NL-A-	8701378	02-01-89
WO-A-9204451	19-03-92	EP-A-	0550428	14-07-93
EP-A-0228726	15-07-87	NL-A-	8503316	16-06-87